

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE PCT NATIONAL STAGE APPLICATION OF

Art Unit: 1627

Coutre, Steven

Examiner: Jean-Louis, Samira JM

INTERNATIONAL APPLICATION NO: PCT/EP2004/006562

FILED: June 17, 2004

U.S. APPLICATION NO: 10/560669

35 USC §371 DATE: March 23, 2007

FOR: Pharmaceutical Uses of Staurosporine Derivatives

Commissioner for Patents  
PO Box 1450  
Alexandria, VA 22313-1450

APPEAL BRIEF

Sir:

This is an appeal from the final rejection of claims 20-39 dated December 17, 2009. The Notice of Appeal was filed on April 16, 2010, making this brief due on June 16, 2010, here extended one month by a simultaneously-filed Petition for Extension of Time to be due on July 16, 2010.

TABLE OF CONTENTS

	page
I. REAL PARTY IN INTEREST	3
II. RELATED APPEALS AND INTERFERENCES	3
III. STATUS OF CLAIMS	3
IV. STATUS OF AMENDMENTS	3
V. SUMMARY OF CLAIMED SUBJECT MATTER	3
VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL	4
VII. ARGUMENT	4
A. THE PRESENT CLAIMS ARE NOT <i>PRIMA FACIE</i> OBVIOUS	4
B. THE EVIDENCE OF RECORD REBUTS ANY <i>PRIMA FACIE</i> OBVIOUSNESS	10
i) THE PRESENT INVENTION SATISFIES A LONG FELT BUT UNMET NEED	10
ii) THE EVIDENCE OF RECORD DEMONSTRATES UNEXPECTED BENEFITS	10
VIII. CONCLUSION	12
CLAIMS APPENDIX	13
EVIDENCE APPENDIX	16
RELATED PROCEEDINGS APPENDIX	49

I. REAL PARTY IN INTEREST

The real party in interest is Novartis AG.

II. RELATED APPEALS AND INTERFERENCES

Appellant is unaware of any related appeals or interferences

III. STATUS OF CLAIMS

Claims 20-39 are pending and claims 1-19 are cancelled. Claims 20-39 are under consideration, rejected and appealed.

IV. STATUS OF AMENDMENTS

The amended claims submitted in the Amendment After Final Rejection filed April 16, 2010, are not entered. The currently pending claims were entered with the Amendment filed on August 6, 2009.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The claims on appeal relate to the treatment of mastocytosis by administering a therapeutically effective amount of the compound of formula VII, which is also known as midostaurin or PKC412.

Independent claim 20 generally claims the treatment of mastocytosis with midostaurin. (Specification at page 1, lines 1-9, and page 31, lines 5-12)

Independent claim 30 specifies the condition to be treated is imatinib-resistant mastocytosis. (Specification at page 31, lines 5-12 and from page 31, line 16, to page 32, line 3)

Claim 34, which depends from claim 30, requires the patient to have a specific mutated KIT tyrosine kinase, with the D186V mutation. (Specification at page 38, lines 17-24)

VI. GROUND OF REJECTION TO BE REVIEWED ON APPEAL

Appellant requests review of all rejections under 35 USC 103(a) on appeal.

Claim 20 was rejected under 35 USC 103(a) over Longley et al (Hematology/Oncology Clinics of North America, Vol. 14, No. 3, pages 689-695) in view of Goekjian et al (Expert Opinions on Investigational Drugs, December 2001, Vol. 10, No. 12, pages 2117-2140).

Claims 30 and 34 were rejected over Longley et al in view of Goekjian et al in further view of Ma et al (Blood, 2002, Vol. 99, No. 5, pages 1741-1744).

Claims 21-28, 31-33 and 35-38 were rejected over Longley et al in view of Goekjian et al in further view of Ma et al and in further view of Caravatti et al (U.S. Patent No. 5,093,330).

Claims 29 and 39 were rejected over Longley et al in view of Goekjian et al in further view of Ma et al and in further view of Caravatti et al in further view of Matthews et al (U.S. Published Application No. 2002/0061873).

VII. ARGUMENT

A. THE PRESENT CLAIMS ARE NOT *PRIMA FACIE* OBVIOUS

Appellant asserts that the present invention is not *prima facie* obvious because the references do not provide a finite number of identified, predictable potential solutions to the problem solved and because the prior art would not lead the skilled artisan to have a reasonable expectation of success.

The present invention provides a method of treating mastocytosis with the compound of formula VII, as set forth in claim 20, which is also known as midostaurin or PKC412. This compound is referred to herein as midostaurin.

The pathogenesis of mastocytosis has been attributed to constitutive activation of the receptor tyrosine kinase, KIT. In a large majority of mastocytosis patients, the deregulated tyrosine kinase activity of KIT is due to a mutation within the codon 816 which results in substitution of Val for Asp816. This mutated KIT is referred to as D816V. This mutation also confers resistance to imatinib, an inhibitor of wild-type KIT that is marketed in the United States under the brand Gleevec® (see, specification at page 38, fifth paragraph, and the paragraph

bridging pages 39 and 40). Appellant points out that claims 30-39 limit the mastocytosis to be treated to imatinib-resistant mastocytosis and that claim 34 requires D816V mutated KIT.

The presently claimed invention is based on the discovery that the mutated form of KIT that is associated with mastocytosis, particularly KIT with the D816V mutation, is effectively inhibited by midostaurin. It was known in the art that midostaurin inhibits wild-type KIT, but it was not known that midostaurin would also inhibit the mutated KIT that is associated with mastocytosis and resistance to imatinib. Appellant asserts that the record does not provide any basis to expect that a compound which inhibits wild-type KIT would inhibit D816V KIT.

Longley et al is relied on by the Examiner as disclosing that activating KIT tyrosine kinase mutations are necessary, if not sufficient, for some forms of mastocytosis and as teaching that inhibiting activated KIT with KIT inhibitors might be therapeutically useful in mastocytosis. However, Longley et al is not alleged to disclose that midostaurin might inhibit such activated KIT or otherwise have utility for the treatment of mastocytosis.

The Examiner relies on Goekjian et al to show that midostaurin is a broad range kinase inhibitor that has been found to inhibit wild-type KIT at approximately micromolar concentrations. Goekjian et al is not alleged to disclose that midostaurin effectively inhibits the activated KIT associated with mastocytosis.

Appellant asserts that Longley et al merely provides a theoretical basis to further experiment with KIT inhibitors for the treatment of mastocytosis. However, Longley et al does not provide any basis to expect that a compound which inhibits wild-type KIT will also inhibit D816V KIT. Indeed, Longley et al provides data that would lead the skilled artisan to understand inhibitors of wild-type KIT are generally not effective inhibitors of D816V KIT.

In support of this position, Appellant points out that the experiments described in Longley et al demonstrate that the KIT inhibitors tested had variable and unpredictable activity against canine P-815 c-KIT, which is taught to correspond to the human D816V mutated KIT. Of the five indolinone KIT inhibitors of wild-type KIT tested by Longley et al, only one, SU6577, at a concentration of 40  $\mu$ M, could substantially inhibit constitutive KIT phosphorylation in the P-815 cell line. The caption under Figure 2 discloses that only SU4984 and SU6577 kill P-815 cells. At page 694, the reference further discusses the variable activity against the activating mutations included in the experiment: "Some compounds can inhibit ligand-induced activation of wild-type receptor but are ineffective against constitutively activated mutants. Other

compounds can inhibit ligand-induced activation of wild-type kit and ligand-independent activation by juxtamembrane domain mutations but not activation by loop mutations."

Appellant asserts that Longley et al would not lead the skilled artisan to generally expect that inhibitors of wild-type KIT would provide a therapeutic benefit in mastocytosis. Indeed, Appellant asserts that Longley et al would lead the skilled artisan to understand that many, if not most, inhibitors of wild-type KIT would not provide a therapeutic benefit in mastocytosis. Thus, it is clear that Longley et al's teaching that inhibiting activated KIT with KIT inhibitors might be therapeutically useful in mastocytosis is merely a general suggestion for further research and is not a suggestion that all KIT inhibitors would be useful for treating mastocytosis. This unpredictability of mutant KIT inhibition is further evidenced by Ma et al, which states that known wild-type KIT inhibitors, ST1571 (also known as imatinib or Gleevec®) and SU9529 did not significantly inhibit D816V mutated KIT. (See the Abstract).

Goekjian et al discloses that midostaurin is an inhibitor of wild-type KIT. However, as discussed above, mere knowledge of a compound's ability to inhibit wild-type KIT does not provide a reasonable expectation of success for the treatment of mastocytosis. Therefore, without further guidance, the combined disclosure of the references would not lead the skilled artisan to have a reasonable expectation of success with respect to midostaurin's potential for treating mastocytosis (claims 20-29), especially mastocytosis that is resistant to imatinib (claims 30-39) and has KIT tyrosine kinase receptor with a D816V mutation (claim 34).

The basis of the rejection is that the combined disclosure of Longley et al with Goekjian et al would make it 'obvious to try' midostaurin for the treatment of mastocytosis. This is clear with respect to the rejection of claims 30 and 34 where the Examiner concludes at page 14 of the final rejection:

Thus, to one of ordinary skill in the art at the time of the invention would have found it obvious to try the midostaurin of Longley in sporadic adult-type mastocytosis since Goekjian teaches that midostaurin is a potent inhibitor of c-KIT with low toxicity properties. Given the teachings of Longley, Goekjian, and Ma, one of ordinary skill would have been motivated to try midostaurin in light of the disclosures of Goekjian, Longley and Ma with the reasonable expectation of providing a method effective in treating sporadic adult type mastocytosis and imatinib-resistant mastocytosis with a potent and low toxicity Staurosporine derivative. (emphasis added)

In response, Appellant asserts that even if, *arguendo*, the claimed invention is 'obvious to try', the Examiner is relying on an improper legal standard for rejecting the present claims. Appellant particularly relies of MPEP 2143(E) and the Federal Circuit's opinion in In Bayer

Schering Pharma AG v. Barr Laboratories, 575 F.3d 1341; 91 U.S.P.Q.2D 1569 (Fed. Cir. 2009), and the case law cited therein, both of which provide guidance about the application an 'obvious to try' standard to reject claims in unpredictable arts. Appellant further asserts that the present rejections under 35 USC 103 are improper based on the standards set forth in MPEP and Bayer v. Barr because what was 'obvious to try' according to the Examiner was merely to explore the general approach of testing KIT inhibitors in mastocytosis without any indication or suggestion that, of all the possible choices, midostaurin would possess the desired properties.

MPEP 2143(E) sets forth the Examiner's burden, according to the USPTO, to properly reject claims based on an 'obvious to try' standard:

**E. "Obvious To Try" - Choosing From a Finite Number of Identified, Predictable Solutions, With a Reasonable Expectation of Success**

To reject a claim based on this rationale, Office personnel must resolve the Graham factual inquiries. Then, Office personnel must articulate the following:

- (1) a finding that at the time of the invention, there had been a recognized problem or need in the art, which may include a design need or market pressure to solve a problem;
- (2) a finding that there had been a finite number of identified, predictable potential solutions to the recognized need or problem;
- (3) a finding that one of ordinary skill in the art could have pursued the known potential solutions with a reasonable expectation of success; and
- (4) whatever additional findings based on the Graham factual inquiries may be necessary, in view of the facts of the case under consideration, to explain a conclusion of obviousness.

Appellant asserts that the present rejection does not satisfy requirements (2) and (3) of the MPEP.

There are numerous compounds identified as KIT kinase inhibitors in the cited references and there are undoubtedly many more KIT inhibitors known in the art. On this basis alone, there are not a finite number of identified possible solutions. However, as discussed above, the evidence of record also provides information that would lead the skilled artisan to expect that many (if not most) of these KIT-inhibiting compounds would not provide a therapeutic benefit against mastocytosis (claims 20-29), and particularly mastocytosis that is resistant to imatinib (claims 30-39) and has KIT tyrosine kinase receptor with a D816V mutation (claim 34). Therefore, it is clear that there are a large number of unpredictable potential solutions, rather than a finite number of identified, predictable solutions, as is required according to MPEP 2143(E)(2).

Moreover, it is clear from the evidence of record that many of the KIT inhibitors tested yielded results that lead to the conclusion that they would not be therapeutically effective against

mastocytosis. It is also clear that the ability to effectively inhibit D816V mutant KIT is expected to be required in order to obtain such a therapeutic benefit. Since none of the cited references provide a basis to expect that midostaurin would effectively inhibit D816V mutant KIT, there is no basis for the skilled artisan to have a reasonable expectation of success, as is required according to MPEP 2143(E)(3).

Appellant's position is further supported by the Federal Circuit's opinion in Bayer v. Barr at 575 F.3d 1347:

In KSR, the Supreme Court stated that an invention may be found obvious if it would have been obvious to a person having ordinary skill to try a course of conduct:

When there is a design need or market pressure to solve a problem and there are a finite number of identified, predictable solutions, a person of ordinary skill has good reason to pursue the known options within his or her technical grasp. If this leads to the anticipated success, it is likely the product not of innovation but of ordinary skill and common sense. In that instance the fact that a combination was obvious to try might show that it was obvious under §103.

550 U.S. at 421. This approach is consistent with our methodology in *In re O'Farrell*, 853 F.2d 894 (Fed. Cir. 1988). See *Procter & Gamble Co. v. Teva Pharms. USA, Inc.*, 566 F.3d 989, 996-97 (Fed. Cir. 2009); *In re Kubin*, 561 F.3d 1351, 1359, (Fed. Cir. 2009). *O'Farrell* observed that most inventions that are obvious were also obvious to try, but found two classes where that rule of thumb did not obtain.

First, an invention would not have been obvious to try when the inventor would have had to try all possibilities in a field unreduced by direction of the prior art. When "what would have been 'obvious to try' would have been to vary all parameters or try each of numerous possible choices until one possibly arrived at a successful result, where the prior art gave either no indication of which parameters were critical or no direction as to which of many possible choices is likely to be successful" an invention would not have been obvious. *O'Farrell*, 853 F.2d at 903. This is another way to express the KSR prong requiring the field of search to be among a "finite number of identified" solutions. 550 U.S. at 421; see also *Procter & Gamble*, 566 F.3d at 996; *Kubin*, 561 F.3d at 1359. It is also consistent with our interpretation that KSR requires the number of options to be "small or easily traversed." *Ortho-McNeil Pharm., Inc. v. Mylan Labs., Inc.*, 520 F.3d 1358, 1364 (Fed. Cir. 2008).

Second, an invention is not obvious to try where vague prior art does not guide an inventor toward a particular solution. A finding of obviousness would not obtain where "what was 'obvious to try' was to explore a new technology or general approach that seemed to be a promising field of experimentation, where the prior art gave only general guidance as to the particular form of the claimed invention or how to achieve it." *O'Farrell*, 853



F.2d at 903. This expresses the same idea as the KSR requirement that the identified solutions be "predictable." 550 U.S. at 421; see also *Procter & Gamble*, 566 F.3d at 996-97; *Kubin*, 561 F.3d at 1359-60.

Appellant asserts that the disclosures of the relied upon references would not lead the skilled artisan to reasonably expect for KIT inhibitors to be generally useful for treating mastocytosis, particularly mastocytosis that is imatinib resistant and has KIT tyrosine kinase receptor with a D816V mutation. Indeed, these references clearly lead the skilled artisan to expect only KIT inhibitors which inhibit the D816V mutant to have the potential for therapeutic utility in most mastocytosis. The references further provide information that would lead the skilled artisan to conclude that many, if not most, KIT inhibitors do not inhibit D816V mutant KIT. Therefore, at best, the combined disclosure of the references merely suggests the general approach of testing KIT inhibitors to determine whether they are useful for treating mastocytosis. However, without some disclosure that would lead the skilled artisan to expect midostaurin to inhibit D816V mutant KIT, it is clear that even if the presently claimed invention is 'obvious to try', it is nevertheless patentable under the case law discussed in Bayer v. Barr. Therefore, Appellant asserts that present rejection is improper and should be reversed.

Appellant further asserts that the additional secondary references do not provide further disclosure which overcomes these problems with rejecting the claims over Longley et al and Goekjian et al.

Claims 30 and 34 were rejected over Longley et al in view of Goekjian et al in further view of Ma et al. Ma et al is relied on as teaching that human mastocytosis is characterized by mutations in KIT codon 816, particularly the D816V mutation, and as disclosing that imatinib (ST1571) does not effectively inhibit this mutated KIT. However, such disclosure does not provide a reasonable basis to expect that midostaurin would inhibit such mutated KIT or be an effective treatment for mastocytosis.

Claims 21-28, 31-33 and 35-38 were rejected over Longley et al in view of Goekjian et al in further view of Ma et al and in further view of Caravatti et al. Caravatti et al is relied on as disclosing dosage forms for treating a patient with midostaurin and the administration of such dosage forms. However, such disclosure does not provide information missing from the other references that would lead the skilled artisan to reasonably expect that midostaurin would be useful for treating mastocytosis.

Claims 29 and 39 were rejected over Longley et al in view of Goekjian et al in further view of Ma et al and in further view of Caravatti et al in further view of Matthews et al. Matthews

et al is relied on as disclosing midostaurin dosage forms that are microemulsions. Again, such disclosure does not provide information missing from the other references that would lead the skilled artisan to reasonably expect that midostaurin would be useful for treating mastocytosis.

**B. THE EVIDENCE OF RECORD REBUTS ANY *PRIMA FACIE* OBVIOUSNESS**

**i) THE PRESENT INVENTION SATISFIES A LONG FELT BUT UNMET NEED**

Although Appellant asserts that the present claims are patentable for the reasons discussed above, Appellant further asserts that the presently claimed invention is patentable because it fills a long felt but unsolved need. See, Graham v. John Deere Co., 383 U.S. 1, 17-18, 86 S. Ct. 684, 15 L. Ed. 2d 545 (1966).

Longley et al discloses the long felt need for effective treatments for mastocytosis. See, page 689-690 and 694: "There is currently no cure for mastocytosis. The treatment of mastocytosis is generally conservative and symptomatic and is designed to prevent or ameliorate the deleterious effects of mast cell mediators rather than to eliminate the mast cells which produce and release them." This view is confirmed by publications made after the filing of the present application: Gotlib et al, Blood 2005, Vol. 106, pages 2865-2870 and Gleixner et al, Blood 2006, Vol. 107, pages 752-759, both which are of record, and attached in the Evidence Appendix. Gotlib et al states in its abstract: "Limited treatment options exist for aggressive systemic mastocytosis (ASM) and mast cell leukemia (MCL)." Gleixner et al indicates at page 752: "However, imatinib failed to inhibit the growth of neoplastic MCs harboring the KIT mutation D816V, which points to a clear need for the identification and development of novel TK inhibitors that block KIT D816V and thus inhibit growth of neoplastic MCs in SM."

The record clearly demonstrates a long felt but unmet need for a therapy to treat mastocytosis. This need is satisfied by the present invention. This secondary indicia of non-obviousness further demonstrates the patentability of the present invention and rebuts any *prima facie* obviousness that might have been established.

**ii) THE EVIDENCE OF RECORD DEMONSTRATES UNEXPECTED BENEFITS**

Appellant further asserts that the Gotlib et al and Gleixner et al publications cited above, as well as Growney et al, Blood, 106(2), 721-724 (2005), which is also of record and in the Evidence Appendix, provide evidence demonstrating that midostaurin possesses properties that

make it more suitable for treating mastocytosis than two other known KIT inhibitors: imatinib and nilotinib (AMN107).

The Growney et al article describes experiments wherein midostaurin is tested against a panel of KIT mutations, including D816V/Y mutations that are associated with systemic mast cell disease (SMCD in the publication), gastrointestinal stromal tumors (GIST) and acute myeloid leukemia (AML). These experiments demonstrate that midostaurin is almost 250 times more active against the D816V KIT mutant than another KIT inhibitor, imatinib. See, page 722, Table 1,  $IC_{50} = 10651$  nM (imatinib) vs. 44 nM (midostaurin). Nothing in the references would lead the skilled artisan to expect midostaurin to be so much more potent than imatinib against the KIT mutation associated with mastocytosis.

The Gleixner et al article describes experiments comparing the D816V mutant KIT inhibiting ability of midostaurin and another known KIT inhibitor, AMN107 or nilotinib, which is now an FDA-approved drug marketed under the brand Tassigna®. The article indicates that midostaurin is a more potent inhibitor of D816V mutant KIT. Midostaurin also inhibited growth in 2 subclones of HMC-1 that either expressed or lacked the D816V mutant KIT and concludes that midostaurin seems to be the first tyrosine kinase inhibitor that counteracts growth of KIT D816V bearing human MCs in the same way as MCs expressing wild type KIT. The article further reports that the growth-inhibitory effects of midostaurin on neoplastic MCs are associated both with inhibition of mutated KIT and with apoptosis. Nothing in the relied upon references would lead the skilled artisan to expect midostaurin to possess such properties. The article concludes that midostaurin seems to be a novel attractive targeted drug worthy to be considered for use in clinical trials in ASM or MCL. See, page 755 right column, first paragraph and the discussion section on pages 757-758.

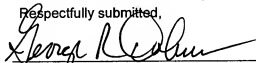
The Gottlib et al article describes a clinical trial involving a patient suffering from several conditions, including systemic mastocytosis. Based on the patient's response to treatment with midostaurin, the authors conclude that midostaurin has promise in the treatment of aggressive forms of systemic mastocytosis.

Appellant asserts that such data demonstrates an unexpected benefit which is sufficient to establish the patentability of the present claims.

VIII. CONCLUSION

Appellant asserts that the several rejections under 35 USC 103 are not proper for the reasons discussed above. Therefore, Appellant respectfully requests that the Board reverse the rejection of the present claims under 35 USC 103.

Respectfully submitted,



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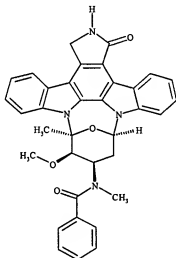
Date: 7/15/2010

CLAIMS APPENDIX

Presented below is a copy of the claims pending in the appeal.

Claims 1-19 (cancelled)

Claim 20. (previously presented) A method of treating mastocytosis, which comprises administering a therapeutically effective amount of the compound of formula (VII)



(VII)

or a pharmaceutically acceptable salt thereof,  
to a human patient suffering from mastocytosis.

Claim 21. (previously presented) A method according to claims 20, wherein the therapeutically effective amount of the compound of formula VII is administered to a mammal subject 7 to 4 times a week or about 100 % to about 50% of the days in the time period, for a period of from one to six weeks, followed by a period of one to three weeks, wherein the agent is not administered and this cycle being repeated for from 1 to several cycles.

Claim 22. (previously presented) A method according to claim 20, wherein the therapeutically effective amount of the compound of formula VII is 100 to 300 mg daily.

Claim 23. (previously presented) A method according to claim 20, wherein the compound of formula VII is administered one, two or three times a day, for a total dose of 100 to 300 mg daily.

Claim 24. (previously presented) A method according to claim 20, wherein the compound of formula VII, is administered three times a day, for a total dose of 225 mg daily.

Claim 25. (previously presented) A method according to claim 20, wherein the compound of formula VII is administered orally.

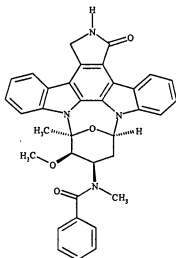
Claim 26. (previously presented) A method according to claim 20, wherein the compound of formula VII is administered as a microemulsion, soft gel or solid dispersion.

Claim 27. (previously presented) A method according to claim 20, wherein up to 150 mg per day of the compound of formula VII is administered.

Claim 28. (previously presented) A method according to claim 23, wherein the compound of formula VII is administered orally.

Claim 29. (previously presented) A method according to claim 28, wherein the compound of formula VII is administered as a microemulsion.

Claim 30. (previously presented) A method of treating mastocytosis with resistance to imatinib, which comprises administering a therapeutically effective amount of the compound of formula (VII)



(VII)

or a pharmaceutically acceptable salt thereof,  
to a patient suffering from mastocytosis with resistance to imatinib.

Claim 31. (previously presented) A method according to claims 30, wherein the therapeutically effective amount of the compound of formula VII is administered to a mammal subject 7 to 4 times a week or about 100 % to about 50% of the days in the time period, for a period of from one to six weeks, followed by a period of one to three weeks, wherein the agent is not administered and this cycle being repeated for from 1 to several cycles.

Claim 32. (previously presented) A method according to claim 30, wherein the therapeutically effective amount of the compound of formula VII is 100 to 300 mg daily.

Claim 33. (previously presented) A method according to claim 30, wherein the compound of formula VII is administered one, two or three times a day, for a total dose of 100 to 300 mg daily.

Claim 34. (previously presented) A method according to claim 30, wherein the patient has KIT tyrosine kinase receptor with a D816V mutation..

Claim 35. (previously presented) A method according to claim 30, wherein the compound of formula VII is administered orally.

Claim 36. (previously presented) A method according to claim 30, wherein the compound of formula VII is administered as a microemulsion, soft gel or solid dispersion.

Claim 37. (previously presented) A method according to claim 30, wherein up to 150 mg per day of the compound of formula VII is administered.

Claim 38. (previously presented) A method according to claim 33, wherein the compound of formula VII is administered orally.

Claim 39. (previously presented) A method according to claim 38, wherein the compound of formula VII is administered as a microemulsion.

EVIDENCE APPENDIX

Appellant provides copies of the following publications which are relied on to demonstrate that the present invention fulfills a long felt but unmet need and to demonstrate that midostaurin possesses unexpected beneficial properties for the treatment of mastocytosis:

- (1) Longley et al., Hematology/Oncology Clinics of North America, Vol. 14, No. 3, pages 689-695
- (2) Ma et al, Blood, 2002, Vol. 99, pages 1741-1744
- (3) Gotlib et al, Blood 2005, Vol. 106, pages 2865-2870
- (4) Gleixner et al, Blood 2006, Vol. 107, pages 752-759
- (5) Growney et al, Blood 2005, Vol. 106, pages 721-724

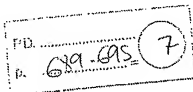
Publication (1) was entered into the record with the Information Disclosure Statement submitted on April 19, 2007.

Publication (2) was cited by the Examiner and entered into the record with the Office action dated May 8, 2009.

Publications 3-5 were entered into the record with the Amendment submitted on August 6, 2009.



XP-008010967

NEW APPROACHES TO THERAPY  
FOR MASTOCYTOSISA Case for Treatment With kit  
Kinase InhibitorsB. J. Longley, MD, Yongsheng Ma, MD, Eric Carter, MD,  
and Gerald McMahon, PhD

## CURRENT THERAPEUTIC OPTIONS

There is currently no cure for mastocytosis. The treatment of mastocytosis is generally conservative and symptomatic and is designed to prevent or ameliorate the deleterious effects of mast cell mediators rather than to eliminate the mast cells which produce and release them.<sup>9</sup> Thus, management of mastocytosis begins with educating the patient to avoid specific factors that can cause the release of mast cell mediators and the symptomatic responses to them. Direct therapeutic intervention is tailored to the symptom complex of individual patients and consists mainly of drugs that block histamine receptors. Blockage of  $H_1$  receptors has some efficacy in controlling cutaneous symptoms such as flushing and itching, and  $H_2$  antagonists are used to prevent and treat histamine-induced peptic ulcer disease, cramping, and diarrhea. Disodium cromoglycate stabilizes mast cell membranes, decreases mediator release, and seems to be helpful in relieving cramping and diarrhea in some patients, but its use is controversial. Besides these extremely aggravating symp-

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toms, which can make life miserable for mastocytosis patients, serious problems such as life-threatening anaphylaxis may be associated with the release of mast cell mediators. Adrenaline and full supportive care may be necessary for anaphylaxis or vascular collapse induced by massive mast cell degranulation. Furthermore, although the contribution of mast cells and their mediators to the pathogenesis of the myelofibrotic and myelodysplastic bone marrow disease, severe anemia, and hematologic malignancy associated with mastocytosis has not been determined, it is possible that the mast cells directly contribute to these life-threatening conditions.

Obviously, it would be preferable to treat mastocytosis by decreasing or eliminating the number of neoplastic mast cells. Several forms of therapy have been used to decrease mast cell numbers temporarily, but these therapies are associated with significant adverse side effects. Psoralen-ultraviolet A therapy (PUVA) can decrease the number of cutaneous mast cells and suppress pruritus. Psoralen-ultraviolet A therapy may also have cosmetic benefits for patients with cutaneous mastocytosis, but its long-term use is associated with an increased risk of skin cancer, whereas its therapeutic benefits are only temporary. Likewise, topical or systemic corticosteroids may transiently decrease the mast cell burden and provide symptomatic relief, but they are associated with long-term cutaneous atrophy, adrenocortical suppression, osteoporosis, and aseptic necrosis of the femoral head, among other side effects. None of these therapies is directed against a specific cause of mastocytosis.

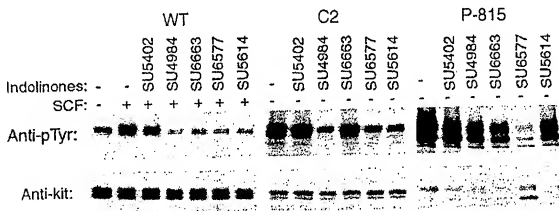
## RATIONALE FOR USE OF KIT INHIBITORS

Recent studies have suggested that mutations affecting the protein coding region of the *c-kit* proto-oncogene may cause some forms of mastocytosis. *c-kit* encodes a receptor tyrosine kinase whose cognate ligand is mast cell growth factor, also known as stem cell factor.<sup>13, 17, 21</sup> Activation of the mast cell growth factor receptor, also known as the kit protein (or simply kit), stimulates mast cell growth and prevents apoptosis.<sup>13</sup> Mutations causing constitutive activation of kit have been found in many mast cell tumor lines and can transform cells both in vitro and in vivo.<sup>4-6, 12, 20</sup> Furthermore, these activating mutations have been found as somatic mutations in the neoplastic mast cells of patients with mastocytosis.<sup>1, 9a, 10</sup> The consistent finding of activating *c-kit* mutations in mast cell tumors, together with the ability of activated kit to stimulate mast cell proliferation and transformation, suggests that these mutations are necessary, if not sufficient, for some forms of mastocytosis and that inhibition of activated kit might be therapeutically useful in mastocytosis. Inhibiting activating kit mutations might provide symptomatic relief by allowing apoptosis of mast cells, thereby decreasing the mast cell load, and might eventually provide a cure by completely eliminating the neoplastic mast cell clone.

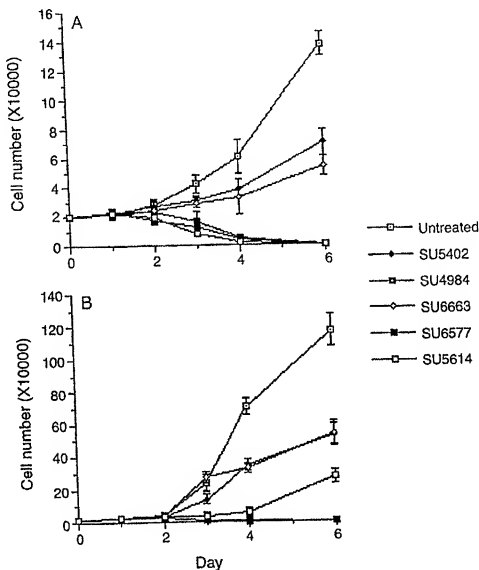
## PRELIMINARY FEASIBILITY STUDIES

Ma et al recently reported testing indolinone derivatives for their ability to inhibit phosphorylation of wild-type and mutated kit in vitro and for their effects on the proliferation and survival of neoplastic mast cell lines expressing activated kit.<sup>12</sup> Indolinone derivatives are polycyclic compounds that inhibit ATP binding in the enzymatic pocket of receptor tyrosine kinases, thereby inhibiting kinase activity.<sup>14, 16</sup> Activating *c-kit* mutations fall into two groups. One group consists of mutations in codon 816 of human *c-kit*, or its equivalent positions in other species, resulting in single-residue substitution for Asp816 in the activation loop of the receptor kinase domain.<sup>10</sup> The other group of activating mutations includes single-residue substitutions and in-frame insertions or deletions in the receptor intracellular juxtamembrane region, which disrupt intramolecular inhibition of the kinase by a putative juxtamembrane  $\alpha$ -helix.<sup>11</sup>

The preliminary studies of Ma et al are described here and reproduced as Figures 1 and 2. These authors found that different indolinones were variably effective at inhibiting wild-type kit and kit with activating mutations. Thus indolinones SU4984, SU6663, SU6577, or SU5614 could all substantially reduce tyrosine phosphorylation of the wild-type receptor at 5- $\mu$ M concentration, whereas SU5402 could only slightly decrease the receptor phosphorylation at this concentration (Fig. 1). Constitutively activated kit mutants expressed by neoplastic mast cells were more resistant to inhibition. For instance, the C2 dog mastocytoma cell line<sup>2, 7</sup> expresses kit that is constitutively active because of a juxtamembrane



**Figure 1.** Effects of indolinone derivatives on phosphorylation of wild-type (WT) and mutant kits. Anti-phosphotyrosine (pTyr) blots of immunoprecipitated WT kit expressed in COS cells and mutant kits expressed in C2 and P-815 cells stimulated (+) or not (-), with stem cell factor (SCF, 100 ng/mL, 10 min) after 30 min incubation (+) or not (-), with indolinone derivatives (5  $\mu$ M for COS and C2 cells, 40  $\mu$ M for P-815 cells) SU4984, SU6663, SU6577, and SU5614 inhibit (WT) kit phosphorylation but variably inhibit C2 and P-815 kit phosphorylation. SU4984, SU6577, and SU5614 are effective against kit containing an activating juxtamembrane domain mutation in C2 cells, while only SU6577 can significantly suppress kit bearing an activating kinase domain mutation in P-815 cells (*top*). Reprobing the anti-pTyr blots (after stripping) with anti-kit antibody shows that comparable amounts of receptor are present in different lanes (*bottom*).<sup>12</sup>



**Figure 2.** Effects of indolinone derivatives on neoplastic mast cell growth. Cell proliferation assay of C2 (A) and P-815 (B) cells treated daily with 1  $\mu$ M and 10  $\mu$ M, respectively, of indolinone derivatives shows that SU4984, SU6577, and SU5614 kill C2 cells, and SU4984 and SU6577 kill P-815 cells. Note that the growth curves of P-815 cells treated with SU4984 and SU6577 overlap. Results are mean, plus or minus the standard error, and represent averages of triplicate cultures.<sup>12</sup>

insertion mutation.<sup>11</sup> Treatment of C2 cells with 5  $\mu$ M of SU4984, SU6577, or SU5614 resulted in an approximately 50% reduction of the constitutive kit phosphorylation (Fig. 1). In contrast, 5  $\mu$ M of SU5402 or SU6663 could barely repress the receptor phosphorylation. P-815 *c-kit* contains a point mutation resulting in substitution of Tyr for Asp814, the equivalent position of human Asp816, which causes constitutive receptor activation in most cases of adult mastocytosis.<sup>10, 90</sup> Activating mutations in the activation loop of the kit kinase domain examined in the P-815 murine mast cell tumor line<sup>3</sup> were even more resistant to inhibition. Among the

five indolinones, only SU6577 at 40  $\mu$ M concentration could substantially reduce the constitutive kit phosphorylation in the P-815 cell line (Fig. 1).

The ability of compounds to inhibit spontaneous kit phosphorylation correlates with their ability to kill mast cells expressing constitutively activated kit. As shown in Figure 2, C2 cells were killed by treating them daily with 1  $\mu$ M of SU4984, SU6577, or SU5614. By comparison, SU5402 and SU6663 only slightly retarded the C2 cell proliferation at this concentration (compare with Fig. 1). The proliferation of P-815 cells containing a kinase domain activation loop mutation was not affected by treating the cells daily with indolinones at 1  $\mu$ M concentration. SU4984 and SU6577, however, killed the P-815 cells at 10  $\mu$ M concentration, whereas SU5402, SU6663, and SU5614 showed only inhibition of P-815 cell proliferation at this concentration (Fig. 2). The reason that P-815 cells are, by an order of magnitude, less susceptible than C2 cells to SU4984 and SU6577 might be related to a difference in the *in vivo* behavior of these two cell lines. P-815 cells are able to form rapidly metastasizing tumors in syngeneic mice and to kill the animals, whereas tumors formed by C2 cells in nude mice do not metastasize and do not kill the host.

It is interesting that the relatively general inhibitor SU4984, although not effective in inhibiting phosphorylation of kit bearing kinase domain activating mutations, can nonetheless kill P-815 cells. The fact that a compound which does not inhibit constitutively activated kit can kill neoplastic mast cells does not detract from the theory that activating *c-kit* mutations are causal events in mastocytosis, because "non-kit" kinases may be involved downstream in the kit signaling pathway, and still other kinases may also be necessary for mast cell survival. Inhibition of these other kinases by SU4984, therefore, may explain its effects on neoplastic mast cell proliferation. Conversely, effective suppression of constitutively activated kit is directly associated with the ability to kill neoplastic mast cells, supporting the hypothesis that activating *c-kit* mutations have a causal role in mastocytosis.<sup>10</sup>

## THERAPEUTIC CONSIDERATIONS

The preliminary *in vitro* results reported by Ma et al indicate that kit kinase inhibitors can effectively kill the neoplastic mast cells which cause some forms of mastocytosis and may eventually form the basis of curative therapy. Even if compounds can be found which are absolutely specific for kit kinase inhibition, they may have side effects which bear consideration. Kit is expressed by other cells besides mast cells, including melanocytes, the interstitial cells of Cajal, germ cells, hematopoietic stem cells, and cells within the central nervous system. Of these cells, the hematopoietic stem cells are perhaps of the greatest concern, because their complete suppression could lead to fatal anemia. Thus, careful studies of kit inhibitors in animals models of mastocytosis will be necessary before therapy can be attempted in humans. Furthermore, although

kit inhibitors may someday prove useful in treating forms of mastocytosis that are not associated with mutations causing constitutive activation, it would be judicious to start clinical trials with patients who have severe, life-threatening disease and who have mastocytosis associated with documented activating c-kit mutations.

## SUMMARY

Some forms of mastocytosis are caused by c-kit mutations which cause constitutive activation of kit kinase. Compounds that inhibit kit kinase, such as indolinones, are therefore attractive as potential therapeutic agents. A hierarchy exists in the ability of compounds to inhibit kit kinase effectively. Some compounds can inhibit ligand-induced activation of wild-type receptor but are ineffective against constitutively activated mutants. Other compounds can inhibit ligand-induced activation of wild-type kit and ligand-independent activation by juxtamembrane domain mutations but not activation by activation loop mutations. Still others effectively inhibit wild-type kit and constitutively activated kit bearing either juxtamembrane or kinase domain mutations and kill the neoplastic mast cells expressing these mutants. No therapy currently exists that specifically targets a cause of mastocytosis, but there are good reasons to believe that kit kinase inhibitors may fulfill that role someday.

## References

1. Buttner C, Henz BM, Welker P, et al: Identification of activating c-kit mutations in adult, but not in childhood-onset indolent mastocytosis: A possible explanation for divergent clinical behavior. *J Invest Dermatol* 111:1227-1231, 1998
2. Devinney R, Gold WV: Establishment of two dog mastocytoma cell lines in continuous culture. *Am J Respir Cell Biol* 3:413-420, 1990
3. Dunn TB, Potter M: A transplantable mast-cell neoplasm in the mouse. *J Natl Cancer Inst* 18:587-601, 1957
4. Furitsu T, Tsujimura T, Tono T, et al: Identification of mutations in the coding sequence of the proto-oncogene c-kit in a human mast cell leukemia cell line causing ligand-independent activation of c-kit product. *J Clin Invest* 92:1736-1744, 1993
5. Hirota S, Isozaki K, Moriyama Y, et al: Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. *Science* 279:577-580, 1998
6. Kitayama H, Kanakura Y, Furitsu T, et al: Constitutively activating mutations of c-kit receptor tyrosine kinase confer factor-independent growth and tumorigenicity of factor-dependent hematopoietic cell lines. *Blood* 85:790-798, 1995
7. Lazarus SC, Devinney R, McCabe LJ, et al: Isolated canine mastocytoma cells: Propagation and characterization of two cell lines. *Am J Physiol* 251:935-944, 1986
8. Ma Y, Longley BJ, Wang X, et al: Clustering of activating mutations in c-KIT's juxtamembrane coding region in canine mast cell neoplasms. *J Invest Dermatol* 112:165-170, 1999
9. Longley BJ, Duffy TP, Kohn S: The mast cell and mast cell disease. *J Am Acad Dermatol* 32:545-561, 1995
- 9a. Longley BJ, Tyrrell L, Lu S-Z, et al: Somatic c-KIT activating mutation in urticaria pigmentosa and aggressive mastocytosis; establishment of clonality in a human mast cell line. *Nat Genet* 12:312-314, 1996

10. Longley BJ Jr, Metcalfe DD, Tharp M, et al: Activating and dominant inactivating c-KIT catalytic domain mutations in distinct clinical forms of human mastocytosis. *Proc Natl Acad Sci U S A* 96:1609-1614, 1999
11. Ma Y, Cunningham ME, Wang X, et al: Inhibition of spontaneous receptor phosphorylation by residues in a putative  $\alpha$ -helix in the KIT intracellular juxtamembrane region. *J Biol Chem* 274:13399-13402, 1999
12. Ma Y, Longley BJ, Carter E, et al: Indolinone derivatives inhibit constitutively activated KIT mutants and kill neoplastic mast cells. *J Invest Dermatol* 114:392-394, 2000
13. Martin FH, Suggs SV, Langley KE, et al: Primary structure and functional expression of rat and human stem cell factor DNAs. *Cell* 63:203-211, 1990
14. Mohammadi M, McMahon G, Sun L, et al: Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. *Science* 276:955-960, 1997
15. Nagata H, Worobec AS, Oh CK, et al: Identification of a point mutation in the catalytic domain of the protooncogene c-kit in peripheral blood mononuclear cells of patients who have mastocytosis with an associated hematologic disorder. *Proc Natl Acad Sci U S A* 92:10560-10564, 1995
16. Piao X, Paulson R, Van Der Geer P, et al: Oncogenic mutation in the Kit receptor tyrosine kinase alters substrate specificity and induces degradation of the protein tyrosine phosphatase SHP-1. *Proc Natl Acad Sci U S A* 93:14665-14669, 1996
17. Qiu F, Ray P, Brown K, et al: Primary structure of c-kit: Relationship with the CSF-1/PDGF receptor kinase family-oncogenic activation of v-kit involves deletion of extracellular domain and C terminus. *EMBO J* 7:1003-1011, 1988
18. Sun L, Tran N, Tang F, et al: Synthesis and biological evaluations of 3-substituted indolin-2-ones: A novel class of tyrosine kinase inhibitors that exhibit selectivity toward particular receptor tyrosine kinases. *J Med Chem* 41:2588-2603, 1998
19. Tsujimura T, Furitsu T, Morimoto M, et al: Ligand-independent activation of c-kit receptor tyrosine kinase in a murine mastocytoma cell line P-815 generated by a point mutation. *Blood* 83:2619-2626, 1994
20. Tsujimura T, Morimoto M, Hashimoto K, et al: Constitutive activation of c-kit in FMA3 murine mastocytoma cells caused by deletion of seven amino acids at the juxtamembrane domain. *Blood* 87:273-283, 1996
21. Yarden Y, Kuang W-J, Yang-Peng T, et al: Human proto-oncogene c-kit: A new cell surface receptor tyrosine kinase for an unidentified ligand. *EMBO J* 6:3341-3351, 1987

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NEOPLASIA

## The c-KIT mutation causing human mastocytosis is resistant to STI571 and other KIT kinase inhibitors; kinases with enzymatic site mutations show different inhibitor sensitivity profiles than wild-type kinases and those with regulatory-type mutations

Yongsheng Ma, Shan Zeng, Dean D. Metcalfe, Cem Akin, Sasa Dimitrijevic, Joseph H. Butterfield, Gerald McMahon, and B. Jack Longley

Mutations of c-KIT causing spontaneous activation of the KIT receptor kinase are associated with sporadic adult human mastocytosis (SAHM) and with human gastrointestinal stromal tumors. We have classified KIT-activating mutations as either "enzymatic site" type (EST) mutations, affecting the structure of the catalytic portion of the kinase, or as "regulatory" type (RT) mutations, affecting regulation of an otherwise normal catalytic site. Using COS cells expressing wild-type or mutant KIT, 2 compounds, STI571 and SU5692, inhibited wild-type and RT mutant KIT at 0.1 to 1  $\mu$ M but did not significantly inhibit the Asp816Val

EST mutant associated with SAHM, even at 10  $\mu$ M. Using 2 subclones of the HMC1 mast cell line, which both express KIT with an identical RT mutation but which differ in that one also expresses the Asp816Val EST mutation, both compounds inhibited the RT mutant KIT, thereby suppressing proliferation and producing apoptosis in the RT mutant-only cell line. Neither compound suppressed activation of Asp816Val EST mutant KIT, and neither produced apoptosis or significantly suppressed proliferation of the cell line expressing the Asp816Val mutation. These studies suggest that currently available KIT inhibitors may be useful in treat-

ing neoplastic cells expressing KIT activated by its natural ligand or by RT activating mutations such as gastrointestinal stromal tumors but that neither compound is likely to be effective against SAHM. Furthermore, these results help establish a general paradigm whereby classification of mutations affecting oncogenic enzymes as RT or EST may be useful in predicting tumor sensitivity or resistance to inhibitory drugs. (Blood. 2002;99:1741-1744)

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### Introduction

The c-KIT protooncogene encodes the KIT protein,<sup>1</sup> the tyrosine kinase receptor for stem cell factor (SCF).<sup>2</sup> KIT is essential for normal development of mast cells in humans and other mammals.<sup>3</sup> Adult-type human mastocytosis is characterized by mutations in c-KIT codon 816, which cause constitutive activation of the KIT kinase.<sup>4,7</sup> A number of mast cell lines and canine mast cell tumors also express activating c-KIT mutations,<sup>4,8,9</sup> and small molecules that inhibit mutant activated KIT effectively kill these cell lines.<sup>10</sup>

In our previous work, we have classified KIT-activating mutations into 2 major groups.<sup>11</sup> One group, exemplified by codon 816 mutations found in human mastocytosis, causes residue substitutions in the activation loop lying at the entrance to the enzymatic pocket.<sup>9</sup> Because these mutations affect the structure of the enzymatic pocket, we have called them "enzymatic pocket" or "enzymatic site" type (EST) mutations. The second group, exemplified by mutations causing residue substitutions, or in-frame deletions or insertions in the intracellular juxtamembrane region found in canine mast cell tumors and in human gastrointestinal stromal

tumors,<sup>9,12</sup> affects the regulation of the activity of an otherwise normal enzymatic site.<sup>13</sup> We have termed these "regulatory" type (RT) mutations. Importantly, different KIT inhibitors vary in their ability to inhibit these different types of mutant KIT and in their ability to inhibit similar types of mutations occurring in different species.<sup>10</sup> Because of these differences, we have proposed that human mastocytosis be classified according to the presence or absence of specific activating mutations and that the ability of a potential therapeutic agent to inhibit the specific activating mutation expressed by a patient's neoplastic mast cells be demonstrated prior to initiation of a therapeutic trial.<sup>14,15</sup>

In this paper, we extend to human mast cells and human KIT our original observations on different classes of activating KIT mutations and their tendency to be inhibited by different pharmacologic agents. Furthermore, we show that not only does the class of mutations affect the sensitivity of activated KIT to inhibition but that specific amino acid substitutions in the same codon may impart differential sensitivity to inhibitors. Because it is relatively easy to

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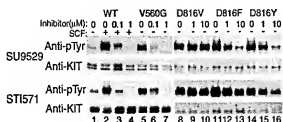
S.D. and G.M. are employed by a company or a competitor of a company whose product was studied in the present work.

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**Figure 1.** Differential sensitivity of RT, EST, and differentially substituted codon 816 mutant KIT to KIT inhibitors. Antiphosphotyrosine blots of immunoprecipitated KIT expressed in COS cells show a low level of spontaneous phosphorylation of wild-type (WT) KIT (lane 1), which increases in response to SCF stimulation (lane 2). Both inhibitors at 0.1 to 1  $\mu$ M prevent this ligand-induced phosphorylation (lanes 3-4). RT mutant KIT with Val560Gy substitution shows a high level of SCF-independent phosphorylation (lane 5), and the phosphorylation is inhibited by both inhibitors at physiologically achievable (0.1-1  $\mu$ M) concentrations (lanes 6-7). The EST Asp816Val mutation commonly found in human mastocytosis also shows high spontaneous phosphorylation (lane 8) but is resistant to inhibition by either KIT inhibitor at 1 to 10  $\mu$ M (lanes 9-10). Substitution of phenylalanine or tyrosine for aspartate 816, rarely found in human mastocytosis, also results in high spontaneous phosphorylation (lanes 11, 14), but these 2 mutant variants respond to the inhibitors at 1 to 10  $\mu$ M (lanes 12-13, 15-16) unlike Asp816Val KIT. However, they are still an order of magnitude less sensitive than are RT mutant or wild-type KIT end are not valid therapeutic targets with currently available drugs.

exclude an EST mutation by sequencing short stretches of tumor DNA and because many clinically available enzyme inhibitors are developed to inhibit the wild-type enzyme, the paradigm we are proposing that separates out mutations that activate by changing the primary structure of the enzymatic site may be useful in understanding mechanisms of drug resistance of other enzymes besides KIT and may help guide therapy in a number of neoplastic diseases.

## Materials and methods

### Cell lines

For these studies we used 2 subclones of the human mast cell leukemia line HMC1.<sup>16</sup> One subclone, HMC1.1, expresses a valine to glycine substitution in codon 560 (Val560Gy) of the KIT intracellular juxtamembrane region. The other, HMC1.2, expresses the Val560Gy mutation and a second substitution, aspartate to valine in codon 816 (Asp816Val) in the kinase enzymatic site. These 2 mutations were originally found together in the HMC1 cell line and were shown to cause SCF-independent constitutive activation of KIT by Furitsu et al.<sup>4</sup> For study of specific KIT mutations, COS cells were used for transient expression of human wild-type and mutated *c-KIT* complementary DNAs, as described previously.<sup>13</sup>

### Inhibitors

Two KIT inhibitors were used. ST1571, manufactured by Novartis (Basel, Switzerland), was developed as an inhibitor of the *c-abl* gene product<sup>17</sup> and has been reported to inhibit wild-type KIT and KIT expressed by HMC1.<sup>18</sup> The other compound, SU9529, is manufactured by SUGEN (San Francisco, CA).

### KIT phosphorylation assay

Cells were serum-starved overnight, incubated with or without inhibitors for 1 hour and with or without SCF (200  $\mu$ g/mL, 10 minutes), followed by immunoprecipitation of cell lysates with anti-KIT antibodies (generously provided by Dr Keith Langley of Amgen, Thousand Oaks, CA), fractionation of proteins by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting with antiphosphotyrosine antibody (Upstate Biotech-

nology, Lake Placid, NY). Blots were stripped and reprobed with anti-KIT antibody as described previously.<sup>13</sup>

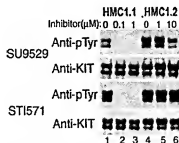
### Cell growth and apoptosis assay

Cells cultured in the presence or absence of inhibitors were counted daily in a hemocytometer using trypan blue exclusion. Proliferation assay was repeated at least 3 times. Apoptosis was examined using DNA fragmentation assay. Briefly, cells were grown in the presence or absence of inhibitors, genomic DNA was isolated and separated by agarose gel electrophoresis, and DNA fragments were visualized under UV light by ethidium bromide staining.

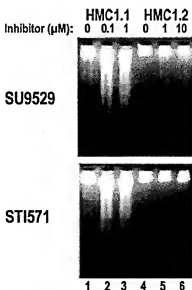
## Results

Both ST1571 and SU9529 prevent the phosphorylation of wild-type KIT induced by its natural ligand SCF and inhibit SCF-independent constitutive phosphorylation of KIT caused by the Val560Gy juxtamembrane RT activating mutation at 0.1 to 1  $\mu$ M (Figure 1). In contrast, both inhibitors fail to inhibit SCF-independent constitutive phosphorylation of KIT containing the Asp816Val EST mutation associated with adult human mastocytosis even at 10  $\mu$ M. Similarly, both drugs inhibit spontaneous KIT phosphorylation in the HMC1.1 subclone (Figure 2, lanes 1-3), which expresses the Val560Gy activating mutation, but at 10  $\mu$ M they fail to inhibit spontaneous phosphorylation of KIT in the HMC1.2 subclone, which expresses both the Val560Gy and Asp816Val activating mutations (Figure 2, lanes 4-6). As would be predicted if the activating mutations caused the proliferation of the mast cells and were necessary for their survival, both inhibitors induce apoptosis of the HMC1.1 cells, causing the death of this line, but fail to kill the HMC1.2 cells (Figures 3 and 4).

Two rare cases of human mastocytosis have been described in which other amino acids besides valine are substituted for Asp816 in the enzymatic site of the KIT kinase.<sup>7</sup> These 2 variants, involving substitution of either tyrosine or phenylalanine, also cause SCF-independent constitutive phosphorylation of KIT. Interestingly, these 2 mutants are partially inhibited by the KIT inhibitors at 1 to 10  $\mu$ M (Figure 1, lanes 11-16), concentrations that are totally ineffective against the most common Asp816Val-substituted mutant (Figure 1, lanes 8-10). Unfortunately, both of these variant EST mutant KITs are an order of magnitude less sensitive than the wild-type or RT mutant KIT, and neither of these inhibitors appear to have a high enough therapeutic index to be valid



**Figure 2.** Differential sensitivity of Val560Gy and Asp816Val mutant KIT in HMC1 subclones to KIT inhibitors. Antiphosphotyrosine blots of immunoprecipitated KIT expressed in 2 HMC1 subclones show that spontaneous phosphorylation of KIT containing only the juxtamembrane RT Val560Gy mutation in the HMC1.1 clone is susceptible to inhibition by both inhibitors at 0.1 to 1  $\mu$ M (lanes 1-3). In contrast, spontaneous phosphorylation of KIT with both the Val560Gy and the EST Asp816Val mutation in the HMC1.2 clone is resistant to both inhibitors at 1 to 10  $\mu$ M (lanes 4-6).



**Figure 3.** Induction of apoptosis in HMC1.1 but not in HMC1.2 cells by KIT inhibitors. DNA fragmentation assay shows that the HMC1.1 clone expressing only the Val560Gly juxta-membrane RT mutation undergoes apoptosis, as indicated by formation of DNA ladders in the presence of the inhibitors at 0.1 to 1 μM (lanes 2-3). In contrast, the HMC1.2 clone expressing both the Val560Gly mutation and the Asp816Val EST mutation does not show any significant DNA ladder in the presence of the inhibitors at 1 to 10 μM (lanes 5-6).

candidates for inhibiting the mutant kinases in a clinical trial. Nevertheless, these data do show that KIT kinases with different residue substitutions in codon 816 of the enzymatic site may show differences in susceptibility to specific pharmacologic inhibitors.

## Discussion

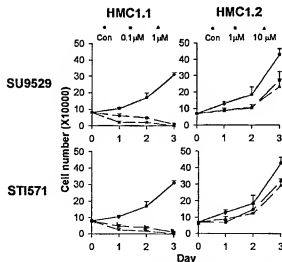
The data presented here show unequivocally that different classes of activating KIT mutations respond differentially to KIT inhibitors. These data extend our previous studies of nonhuman mammalian KIT-activating mutations to the actual mutations found in various forms of human disease and support our proposals for classification of mutations as RT or EST mutations and for classification of human disease according to the type of the mutations expressed in specific tumors.<sup>10,11,14,15</sup> Our current results also highlight the need to identify specific variants of mutant KIT expressed by individual patients when one contemplates rational therapy.

It appears likely that activating mutations affecting other enzymes may also be classified as regulatory or enzymatic site in type and that this paradigm may prove to be generally useful in predicting drug resistance and guiding therapy. A recent study by Gorre et al<sup>19</sup> supports this prediction. In the study, the authors report that the development of resistance to STI571 in 6 of 9 patients with BCR-ABL-positive chronic myeloid leukemia was associated with acquisition of mutations that directly affected the active site of the enzyme, and resistance in the other 3 patients was associated with BCR-ABL gene amplification. We would classify the former as EST mutations and the gene amplification (as well as the original translocation forming the BCR-ABL fusion gene) as RT mutations or events. Gorre et al suggest that the patients with

gene amplification might be susceptible to treatment with higher doses of STI571 but that those with active site mutations may require treatment with a different drug, a speculation with which we agree. Although their study may be viewed as retrospective in nature, the association of resistance in a previously sensitive tumor with the acquisition of an EST mutation supports the general clinical utility of the paradigm we are proposing.

The incidental finding that different amino acid substitutions in codon 816 of the enzymatic site give rise to differential sensitivity to drugs is not unexpected, but it represents the first documentation with human mutant activated KIT to support individualization of therapy based on the response of specific mutant proteins to specific drugs. Accordingly, our data suggest that despite the previously reported ability of the KIT inhibitor STI571 to kill an HMC1 line at 0.1 μM,<sup>18</sup> currently available KIT inhibitors may be ineffective in treating human adult-type mastocytosis. On the other hand, neoplastic processes characterized by RT KIT-activating mutations, such as gastrointestinal stromal tumors, should be susceptible to inhibition by a relatively wide variety of inhibitors, including those that inhibit wild-type KIT. It has been our experience that different RT mutations, in a given species, show similar sensitivities regardless of the specific amino acid substitution<sup>10</sup> (additional data not shown). This observation supports the concept that the enzymatic site in our proposed RT mutants does not differ significantly from the enzymatic site of wild-type KIT. It follows that a drug that is a "good fit" for the wild-type enzymatic site and is capable of sterically blocking the enzymatic reaction would be likely to be also effective against a RT mutant but would not necessarily be effective against an EST mutant. This concept may aid in identifying potentially clinically useful drugs.

The data presented also shed a unique perspective on the cause of mast cell neoplasms. The fact that the HMC1.1 and 1.2 cell lines are only known to differ by the presence or absence of the Asp816Val mutation makes them an ideal model for determining the role of KIT activation in the factor-independent growth and survival of these cells. The key observation is the fact that both drugs inhibit the RT activating KIT mutation, and both drugs are



**Figure 4.** Differential effects of KIT inhibitors on the growth of HMC1.1 and HMC1.2 cells. Cell proliferation assay shows that incubation of HMC1.1 cells with inhibitors at 0.1 to 1 μM for a 3-day period kills the cells, while treatment of the HMC1.2 cells with inhibitors at 1 to 10 μM only slightly inhibits growth of the cells.

capable of killing the HMC1.1 cell line, which expresses only that mutation. However, neither of these drugs are capable of inhibiting the EST mutation found in the HMC1.2 clone, and neither are capable of inhibiting the growth and survival of that cell line. Together, these observations show that the ability to kill neoplastic

mast cells expressing activated KIT is associated with the ability to inhibit the mutated, activated KIT rather than to inhibit some other unknown kinases. Thus, this finding strongly supports the hypothesis that it is the mutated, activated KIT molecule itself that is the cause of adult-type mastocytosis.

## References

- Yarden Y, Kuang WJ, Yang-Feng T, et al. Human proto-oncogene c-kit: a new cell surface receptor tyrosine kinase for an unidentified ligand. *EMBO J*. 1987;6:3341-3351.
- Martin FH, Suggs SV, Langley KE, et al. Primary structure and functional expression of rat and human stem cell factor DNAs. *Cell*. 1990;63:203-211.
- Galli SJ, Zsebo KM, Geissler EN. The kit ligand, stem cell factor. *Adv Immunol*. 1994;55:1-98.
- Furuta T, Tsujimura T, Tono T, et al. Identification of mutations in the coding sequence of the proto-oncogene c-kit in a human mast cell leukemia cell line causing ligand-independent activation of c-KIT product. *J Clin Invest*. 1993;92:1736-1744.
- Nagata H, Worobec AS, Oh CK, et al. Identification of a point mutation in the catalytic domain of the proto-oncogene c-kit in peripheral blood mononuclear cells of patients who have mastocytosis with an associated hematologic disorder. *Proc Natl Acad Sci U S A*. 1995;92:10560-10564.
- Longley BJ, Tyrrell L, Lu S, et al. Somatic c-KIT activating mutation in urticaria pigmentosa and aggressive mastocytosis: establishment of clonality in a human mast cell neoplasm. *Nat Genet*. 1996;12:312-314.
- Longley BJ, Metcalfe DD, Tharp M, et al. Activating and dominant inactivating c-KIT catalytic domain mutations in distinct clinical forms of human mastocytosis. *Proc Natl Acad Sci U S A*. 1999;96:1603-1614.
- Tsujimura T, Furuta T, Morimoto M, et al. Ligand-independent activation of c-kit receptor tyrosine kinase in a murine mastocytoma cell line p815 generated by a point mutation. *Blood*. 1994;83:2819-2826.
- Ma Y, Longley BJ, Wang X, et al. Clustering of activating mutations in c-KIT's juxtamembrane coding region in canine mast cell neoplasms. *J Invest Dermatol*. 1999;112:165-170.
- Ma Y, Carter E, Wang X, et al. Indolirone derivatives inhibit constitutively activated KIT mutants and kill neoplastic mast cells. *J Invest Dermatol*. 2000;114:392-394.
- Longley BJ, Reguera MJ, Ma Y. Classes of c-KIT activating mutations: proposed mechanisms of action and implications for disease classification and therapy. *Leuk Res*. 2001;25:571-578.
- Hirota S, Isozaki K, Moriyama Y, et al. Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. *Science*. 1998;279:577-580.
- Ma Y, Cunningham M, Wang X, et al. Inhibition of spontaneous receptor phosphorylation by residues in a putative  $\alpha$ -helix in the KIT intracellular juxtamembrane region. *J Biol Chem*. 1999;274:13399-13402.
- Longley BJ, Metcalfe DD. A proposed classification for mastocytosis incorporating molecular genetics. *Hematol Oncol Clin North Am*. 2000;14:697-701.
- Longley BJ, Ma Y, Carter E, et al. New approaches to therapy for mastocytosis: a case for treatment with kit kinase inhibitors. *Hematol Oncol Clin North Am*. 2000;14:689-695.
- Butterfield JH, Weller D, Dewald G, et al. Establishment of an immature mast cell line from a patient with mast cell leukemia. *Leuk Res*. 1988;12:345-355.
- Buchdunger E, Zimmermann J, Mett H, et al. Inhibition of the Abl protein-tyrosine kinase in vitro and in vivo by a 2-phenylaminopyrimidine derivative. *Cancer Res*. 1996;56:100-104.
- Heinrich MC, Griffith DJ, Druker BJ, et al. Inhibition of c-kit receptor tyrosine kinase activity by STI 571, a selective tyrosine kinase inhibitor. *Blood*. 2000;96:925-932.
- Gomez ME, Mohammed M, Ellwood K, et al. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science*. 2001;293:876-880.

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## Brief report

# Activity of the tyrosine kinase inhibitor PKC412 in a patient with mast cell leukemia with the D816V *KIT* mutation

Jason Gotlib, Caroline Berubé, Joseph D. Gowney, Ching-Cheng Chen, Tracy I. George, Christopher Williams, Tomohiro Kajiguchi, Jia Ruan, Stan L. Lillberg, Jeffrey A. Durocher, Jack H. Lichy, Yanfeng Wang, Pamela S. Cohen, Daniel A. Arber, Michael C. Heinrich, Len Neckers, Stephen J. Galli, D. Gary Gilliland, and Steven E. Coutre

The majority of patients with systemic mast cell disease express the *Imatinib-resistant* Asp816Val (D816V) mutation in the *KIT* receptor tyrosine kinase. Limited treatment options exist for aggressive systemic mastocytosis (ASM) and mast cell leukemia (MCL). We evaluated whether PKC412, a small-molecule inhibitor of *KIT* with a different chemical structure from imatinib, may have therapeutic use in advanced SM with the D816V *KIT* mutation. We treated a patient with MCL (with an associated myelodysplastic syn-

drome (MDS)/myeloproliferative disorder (MPD) based on *in vitro* studies demonstrating that PKC412 could inhibit D816V *KIT*-transformed Ba/F3 cell growth with a 50% inhibitory concentration ( $IC_{50}$ ) of 30 nM to 40 nM. The patient exhibited a partial response with significant resolution of liver function abnormalities. In addition, PKC412 treatment resulted in a significant decline in the percentage of peripheral blood mast cells and serum histamine level and was associated with a decrease in *KIT* phosphorylation and

D816V *KIT* mutation frequency. The patient died after 3 months of therapy due to progression of her MDS/MPD to acute myeloid leukemia (AML). This case indicates that *KIT* tyrosine kinase inhibition is a feasible approach in SM, but single-agent clinical efficacy may be limited by clonal evolution in the advanced leukemic phase of this disease. (Blood. 2005; 106:2865-2870)

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## Introduction

Mastocytosis comprises a spectrum of disorders related to the abnormal growth and accumulation of mast cells in one or more organs. The World Health Organization (WHO) recognizes 4 systemic mastocytosis (SM) subtypes: indolent SM (ISM), SM with associated clonal hematologic non-mast-cell lineage disease (SM-AHNMD), aggressive SM (ASM), and mast cell leukemia (MCL).<sup>1</sup> The AHNMD typically consists of a myelodysplastic syndrome (MDS), chronic myeloproliferative disorder (MPD), overlap MDS/MPD, or acute myelogenous leukemia.<sup>2</sup>

The proto-oncogene *KIT* encodes a transmembrane receptor tyrosine kinase that is expressed on mast cells and other hematopo-

ietic lineages.<sup>3</sup> A pathogenetic hallmark of the majority of SM cases in adults is the Asp816Val (D816V) somatic mutation in the catalytic domain of the *KIT* gene.<sup>4-6</sup> This transforming mutation results in enhanced mast cell survival and proliferation because of constitutive activation of the tyrosine kinase activity of *KIT*, independent of *KIT* ligand.<sup>6</sup>

The management of patients with SM involves attempting to control symptoms related to mediator release from mast cells and to curtail organ damage caused by infiltrating mast cells.<sup>2</sup> Advanced mast cell disease (eg, ASM and MCL) carries a poor prognosis. Current treatments such as interferon-alpha with or without corticosteroids

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P.S.C. and Y.W. are employed by a company (Novartis Pharmaceuticals) whose product was studied in the present work.

J.G. participated in the writing of the manuscript, design of the clinical trial protocol, treatment of the patient, and interpretation of the data; C.B., J.R., and S.E.C. participated in treatment of the patient; J.D.G. performed the *in vitro* cell

growth inhibition studies and *KIT* immunoprecipitation experiments with PKC412; D.G.G. participated in the experimental design of these studies and provided the *in vitro* data to support the use of PKC412 in this patient; C.-C.C. and S.J.G. performed sequencing of cloned RT-PCR products containing the *KIT* sequence; S.J.G. also contributed to the writing of the manuscript and interpretation of the data; Y.W. performed the pharmacokinetic studies; P.S.C. provided PKC412 for the *in vitro* studies and treatment of the patient and contributed to writing of the manuscript; S.L.L. and J.A.D. performed semiquantitative D816V *KIT* mutation analysis and screening of all *KIT* exons and interpretation of these data; J.H.L. performed initial direct sequencing of the *KIT* gene from genomic DNA before and during PKC412 therapy; T.I.G. and D.A.A. performed the immunohistochemical and flow cytometric analysis of patient samples and contributed to writing of the manuscript; L.N. generated the experimental design for the *KIT* phosphorylation studies; T.K. and C.W. performed the *KIT* phosphorylation experiments; and M.C.H. performed *in vitro* analysis of the effects of PKC412 on D816V-transformed cell growth, serving as the rationale for treatment of this patient with PKC412.

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and cladribine exhibit low response rates that are usually partial in nature.<sup>7,9</sup> The D816V *KIT* mutation of SM has been shown to be resistant to the tyrosine kinase inhibitor imatinib mesylate (Gleevec) both in vitro and in vivo.<sup>10-12</sup> We therefore evaluated the effects of PKC412 (*N*-benzoyl-staurosporine, Novartis, Basel Switzerland), an alternative small molecule inhibitor of multiple type III receptor tyrosine kinases, including the *KIT* tyrosine kinase, in a patient with mast cell leukemia.

## Study design

In April 2003, a previously healthy 48-year-old woman presented with a 4-week history of malaise, fever, night sweats, and rash. Notable physical findings included 3-cm palpable splenomegaly, a diffuse petechial rash, and scattered less than 1.0 cm hyperpigmented macular lesions on the trunk and extremities. Biopsy of the skin lesions revealed a mast cell infiltrate with no definite staining of mast cells by CD25. Table 1 shows laboratory studies at initial presentation. The peripheral blood was remarkable for an increase in myeloid immaturity and 11% circulating mast cells. A bone marrow aspirate revealed 8% myeloblasts, trilineage dysplasia, and markedly decreased megakaryocytes. The bone marrow biopsy revealed 90% to 100% cellularity, with mast cells accounting for 70% of marrow cellularity. Spindled-cell aggregates of mast cells (Figure 1B-C) were highlighted by immunohistochemical staining for CD117, mast cell tryptase, and CD25 (Figure 1D-E), but were negative for CD2 by flow cytometry immunophenotyping. Testing of the bone marrow aspirate revealed heterozygosity for the D816V *KIT* mutation. Cytogenetic analysis showed a normal female karyotype and polymerase chain reaction (PCR) for *BCR-ABL* was negative. The findings were consistent with MCL with an AHNMD, MDS/MPD.

During the following 2 months, the patient's disease progressed with 30% to 50% circulating mast cells. The patient was supported with red

blood cell and platelet transfusions, famotidine, lansoprazole, lorazepam, and cromolyn sodium for mediator-related symptoms, including pruritis and persistent diarrhea. She developed progressive liver dysfunction, severe ascites, and a portal vein thrombosis. She was hospitalized for marked deterioration of her clinical status. She declined treatment with alpha-interferon. Findings of a repeat bone marrow biopsy were similar to the specimen at initial presentation. The patient agreed to participate in a compassionate use trial of PKC412. The Stanford Medical Center institutional review board approved the study, and the patient gave written informed consent according to the Declaration of Helsinki. PKC412 was initiated in June 2003 at 100 mg twice daily as 28-day cycles, the recommended dose from phase 1 studies in acute myelogenous leukemia (AML). The patient was also treated with furosemide, spirinolactone, and tincture of opium for diarrhea.

## Histology and immunophenotyping studies

For immunohistochemistry, antibodies included CD25 (4C9; Novocastra, Newcastle upon Tyne, United Kingdom), CD34 (My10) (Becton Dickinson, San Jose, CA); CD117 (KIT, Dako, Carpinteria, CA), and mast-cell tryptase (G3, Chemicon, Temecula, CA). Direct dual-parameter flow cytometry was performed on a FACSCalibur instrument (Becton Dickinson). Fluorescein isothiocyanate, phycoerythrin, and tricolor-conjugated monoclonal antibodies were used: CD45 (H130; Caltag, Burlingame, CA); CD34 (581) CD64 (22) (Immunotech, Marseille, France); CD2 (SS2), CD117 (104D2), and CD25 (2A3) (Becton Dickinson).

## PKC412 pharmacokinetics (PK)

Blood samples were collected at predose and during treatment. Plasma concentrations of PKC412 and its major active metabolite CGP62221 were determined using a high performance liquid chromatography method with fluorescence detection, with a lower limit of quantification of 5.1 ng/mL.<sup>13</sup>

Table 1. Patient laboratory values before and during treatment with PKC412

	Initial Presentation	Pretreatment	Cycle 1	Cycle 2	Cycle 3/Relapse
Date	Apr 2003	Jun 2003	Jun-Jul 2003	Jul-Aug 2003	Aug-Sep 2003
<b>Hematology</b>					
WBCs/mm <sup>3</sup> *	15.8	8.4	8.0	7.9	12.2
Differential count, %					
Segmented neutrophils	14	19	48	44	20
Bands	10	6	29	26	7
Lymphocytes	19	19	11	17	7
Monocytes	8	5	7	8	0
Eosinophils	0	2	0	1	0
Basophils	0	0	0	0	0
Immature myeloids	31	3	2	4	51
Blasts	5	0	0	0	8
Mast cells	11	46	5	0	2
Nucleated red blood cells/100 WBCs	7	47	4	4	0
Hemoglobin, g/dL†	9.8	8.5	8.7	7.5	9.5
Hematocrit, %	29.8	26.6	25.8	22.8	31.0
Platelets/mm <sup>3</sup> ‡	5000	7000	13 000	16 000	11 000
<b>Chemistry</b>					
Albumin, g/dL†	2.4	2.2	2.8	3.4	2.3
Total bilirubin, mg/dL§	1.2	4.8	2.1	1.3	13.6
Direct bilirubin, mg/dL§	0.5	2.8	1.1	0.7	7.5
LDH, U/L	552	789	239	227	595
<b>Mastocytosis-related findings</b>					
Serum histamine, ng/dL	ND	6910	1031	779	2525
Serum tryptase, µg/L	> 200	> 200	> 200	> 200	> 200

WBCs indicates white blood cells; LDH, lactate dehydrogenase.

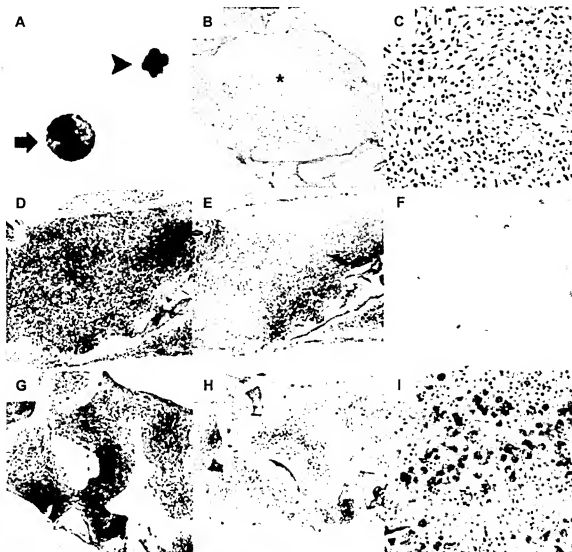
\*To convert to  $\times 10^9$  cells per liter, multiply by 1.

†To convert to grams per liter, multiply by 10.

‡To convert to  $\times 10^9$  platelets per liter, divide by 1000.

§To convert to micromoles per liter, multiply by 17.1.

|Transfusion-dependent.



**Figure 1.** Peripheral blood and bone marrow findings before and after treatment with PKC 412. (A-F) Before treatment. (G-I) After treatment. (A) Circulating mast cell (arrow, left) and dysplastic nucleated red blood cell (arrowhead, right) in peripheral blood. Wright-Giemsa, 1000 $\times$ . (B) Hypercellular bone marrow with aggregates of pale-staining mast cells surrounding a dilated sinus (\*). Hematoxylin and eosin, 40 $\times$ . (C) Whorled nodule of mast cells with characteristic clear cytoplasm including spindled forms. Hematoxylin and eosin, 400 $\times$ . (D) Increased mast cells show a nodular and interstitial pattern in the bone marrow occupying approximately 70% marrow cellularity. Mast cell tryptase, 40 $\times$ . (E) The majority of mast cells are highlighted by CD25 antibody. CD25, 40 $\times$ . (F) Few numbers of CD34-positive blasts ( $\sim$ 5%) detected on bone marrow biopsy. CD34, 400 $\times$ . (G) The posttreatment bone marrow shows a similar mast cell burden as seen prior to treatment. Mast cell tryptase, 40 $\times$ . (H) A slight decrease in CD25-positive mast cells ( $\sim$ 40% of marrow cellularity) is noted after therapy. CD25, 40 $\times$ . (I) Increased numbers of CD34-positive blasts (10%-20%) in bone marrow biopsy indicating progression of this patient's AHNMD. MDS/MPD. CD34, 400 $\times$ .

#### DNA constructs, cell cultures, and cell growth inhibition studies

The D816V mutation of human *KIT* (lacking amino acids 510-513, GNNK) in pCDNA3 was generated (Transformer Site-Directed Mutagenesis kit, Clontech, Mountain View, CA), then subcloned into the *Xho*I site of retroviral vector MSCV-IRES-GFP. Retroviral transduced Ba/F3 cells were selected for interleukin-3 (IL-3) independent growth.  $1 \times 10^4$  cells were plated in triplicate in 96 well plates with or without indicated concentrations of the drug and the absence of IL-3. After 24 hours,  $1 \mu$ Ci (0.037 MBq)  $^3$ H-thymidine was added, followed by a 4-hour incubation. Cells were harvested and  $^3$ H-thymidine incorporations determined.

#### PCR analysis of the *KIT* gene

Genomic DNA (gDNA) was isolated from ficoll peripheral blood or bone marrow mononuclear cell specimens (200  $\mu$ L) with the QIAamp DNA blood minikit (Qiagen, Valencia, CA). PCR of the exon 17 region of the *KIT* gene containing the site of the D816V mutation was performed with the

primer sequences *KIT*17S: TGGCAGCCAGAAATATCTCTC and *KIT*17AS: CACGGAAACAATTTTATCGAA. Amplification was carried out on an ABI 9600 thermocycler. The 182-nucleotide (nt) PCR product was purified after electrophoresis on a 2% agarose gel. Sequencing reactions were analyzed on an ABI Prism 377 DNA Sequencer.

RNA (1  $\mu$ g) from ficoll bone marrow samples was isolated using an RNeasy minikit (Qiagen, Valencia, CA). First-strand cDNA was generated using oligo(dT)<sub>12-18</sub> primers (Promega, Madison, WI) and Omniscript reverse transcriptase (Qiagen) before amplification with PCR *KIT*13: GACGAGTTGGCCCTAGAC and PCR *KIT*14: AGTTGGAGTAAATAT-GATTGGTG using PfuTurbo polymerase (Stratagene, La Jolla, CA).<sup>14</sup> The resulting 538-base pair (bp) fragment was gel purified then cloned using Zero Blunt TOPO PCR cloning kit (Invitrogen, Carlsbad, CA). For each cloning, 3 positive clones were selected for sequencing (by SequenTech, Mountain View, CA) at both strands.

In-depth mutational analysis of all *KIT* exons (1-21) and the corresponding 30 bp of flanking intronic sequence was conducted using fluorescent

denaturing high-performance liquid chromatography (DHPLC) technology<sup>15</sup> and Surveyor mismatch cleavage analysis<sup>16</sup> both with the WAVE HS System (Transgenomic, Omaha, NE). Purified gDNAs (5–10 ng) were subjected to 35 cycles of PCR using Optima, a proofreading DNA polymerase (Transgenomic) and primer pairs for each *KIT* amplicon. Negative gDNA controls were included in the amplifications. Aliquots (3–15  $\mu$ L) were scanned for mutations by DHPLC, confirmed by Surveyor mismatch cleavage, and identified with bidirectional sequence analysis on an ABI 3100 sequencer using BigDye terminator chemistry (Applied Biosystems, Foster City, CA). For semiquantitative determination of mutant and normal allele frequencies, relative peak areas of DHPLC elution profiles and Surveyor mismatch cleavage products were determined after normalization and comparison to reference controls using the WAVE Navigator software.

The sensitivity of the DHPLC assay as compared to direct DNA sequencing was determined by mixing a mast cell leukemia cell-line (HMC-1) containing the *KIT* D816V mutation<sup>17</sup> with wild-type *KIT* cells at mutant allele frequencies of 0.5%, 0.75%, 1%, 2%, 5%, 7.5%, 10%, 20%, 50%, and 90%. Genomic DNA was extracted from each sample as described, and mutation analysis was performed in triplicate. The D816V *KIT* mutation was confirmed by DHPLC in all replicates down to an allelic frequency of 1%, as compared to 7.5% for DNA sequencing.

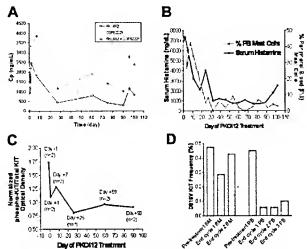
#### Ex vivo analysis of KIT phosphorylation during PKC412 treatment

Cell lysates were prepared from frozen peripheral blood and bone marrow aspirates of the patient before and after treatment with PKC412. KIT protein immunoprecipitation was performed using a cocktail of anti-KIT antibodies (M-14, C-19, and Ab81; all from Santa Cruz Biotechnology, Santa Cruz, CA), as previously described.<sup>17</sup> For Western blot analysis, total KIT protein was detected with anti-KIT antibody C-19 (Santa Cruz Biotechnology), and phosphorylated KIT protein was visualized by probing duplicate blots with a phospho-KIT-specific antibody (Tyr719; Cell Signaling Technology, Beverly, MA).

## Results and discussion

Table 1 lists relevant abnormal laboratory studies at the start of treatment, best responses during subsequent 28-day cycles of PKC412 treatment, and findings at disease progression. During cycle 1, the patient's Karnofsky performance status improved from 20% to 70%. She experienced improvement in her diarrhea and ascites, and her portal vein thrombosis recanalized. Mild reduction of splenomegaly was noted on physical examination. The percentage of peripheral blood mast cells decreased from 46% to 5% (Figure 2B), accompanied by increasing myeloid maturity (Table 1). The total/direct bilirubin decreased from 82.08/47.88  $\mu$ M (4.8/2.8 mg/dL) to 35.91/18.81  $\mu$ M (2.1/1.1 mg/dL), and the lactate dehydrogenase (LDH) decreased from 769 to 239 U/L. The serum histamine decreased from 6910 ng/dL to 1031 ng/dL (Figure 2B), but the serum tryptase remained more than 200  $\mu$ g/L. A bone marrow biopsy at the end of cycle 1 showed a decrease in myeloblasts to lower than 5%, but there was persistent dysplasia and both interstitial and nodular aggregates of mast cells, comprising 70% of the marrow cellularity (similar to baseline).

During the second cycle of PKC412, the peripheral blood mast cell count was undetectable or below 5% (Figure 2B), myeloid maturity persisted, and there was a 2-week period of platelet transfusion independence. The serum histamine decreased further, to 779 ng/dL (Figure 2B). The total/direct bilirubin almost normalized to 22.23/11.97  $\mu$ M (1.30/0.7 mg/dL). At the end of cycle 2, a repeat bone marrow biopsy showed a decrease in the proportion of CD25<sup>+</sup> mast cells to 40% of marrow cellularity (Figure 1H) and an



**Figure 2.** Changes in the pharmacokinetics of PKC412 and patient mast cell disease-related parameters during PKC412 therapy. (A) Trough plasma concentration-time profiles of PKC412 and its major active metabolite CGP62221 and the sum of PKC412 and CGP62221 in the mast cell leukemia patient receiving PKC412 100 mg twice a day between day 1 and day 90, and 75 mg three times a day starting from day 90. (B) Serum histamine level and percent peripheral blood mast cells quantified by manual differential. (C) Normalized phospho-KIT/total KIT optical density ratio. The number of samples analyzed at each time point is shown in parentheses. (D) Semiquantitative DHPLC determination of the D816V KIT mutation frequency in the bone marrow and peripheral blood.

increase in myeloblasts to 10% to 20% of marrow cellularity by CD34 immunohistochemical staining (Figure 1D). During cycle 3 of treatment, the patient exhibited signs of disease progression. Laboratory findings included an increasing white blood cell (WBC) count with reappearance of myeloid immaturity and 5% to 10% myeloblasts in the peripheral blood and loss of platelet transfusion independence. Despite an increase in the PKC412 dose to 75 mg 3 times daily, the patient experienced deterioration of her performance status in conjunction with progressive liver failure. The patient expired on day 111 after initiation of PKC412 treatment. At the time of disease progression, peripheral blood mast cells generally remained less than 5% (Figure 2B).

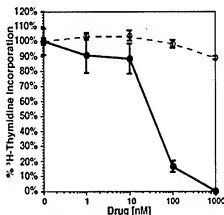
#### Side effects of PKC412

PKC412 was generally well tolerated. The patient experienced grade 1 to 2 nausea and vomiting (National Cancer Institute version 3.0 Common Terminology Criteria for Adverse Events)<sup>18</sup> with a suspected relationship to PKC412. Grade 4 hyperbilirubinemia developed during relapse, which was considered unlikely related to PKC412 since the patient presented with liver disease.

#### Pharmacokinetics

PKC412 trough plasma concentrations increased in the first 3 days and reached a maximum trough concentration of 2450 ng/mL on day 3, and then started to decline slowly and reached a new steady-state concentration of approximately 450 ng/mL on day 28 (Figure 2A). It appeared that the plasma concentration of PKC412 showed a trend of decrease in cycle 3 (days 60 to 90) until the dose was increased on day 90. On day 90, PKC412 and CGP62221 concentrations were 297 ng/mL and 730 ng/mL, respectively. After the PKC412 dose was increased from 100 mg twice a day to 75 mg three times a day, both PKC412 and CGP62221 concentrations were elevated substantially.





**Figure 3.** Ba/F3-KIT D816V is sensitive to PKC412 but not to imatinib. Plotted is the percentage of <sup>3</sup>H-thymidine incorporation in drug-treated wells relative to no drug controls. Cells were treated with imatinib (dashed line, ○) or PKC412 (solid line, ●) for 24 hours in the presence of 10 ng/mL IL-3 and the absence of recombinant human stem cell factor (SCF). Plotted is the percent <sup>3</sup>H-thymidine incorporation in drug-treated wells relative to no drug controls. Data are the mean of triplicates (plot standard deviation). PKC412 IC<sub>50</sub> 30 nM–40 nM.

#### In vitro analysis of the effects of PKC412 on D816V KIT-transformed cell growth

The D816V *KIT* mutant stably transformed Ba/F3 cells to IL-3-independent growth (data not shown). As measured by <sup>3</sup>H-thymidine incorporation, D816V KIT-transformed Ba/F3 cells were resistant to treatment with imatinib (Figure 3). In contrast, PKC412 effectively inhibited the growth of D816V KIT-transformed Ba/F3 cells with a cellular 50% inhibitory concentration (IC<sub>50</sub>) of approximately 30 nM to 40 nM (Figure 3). Using Western blot analysis of lysates from PKC412-treated Ba/F3 cells, we (J.D.G. and D.G.G.) recently demonstrated reduction of the phosphotyrosine content of both c-KIT and the downstream effector STAT3, but not STAT5.<sup>19</sup> Previous reports have shown that STAT3 is aberrantly phosphorylated by D816V KIT.<sup>20,21</sup>

#### PCR of the *KIT* gene

Using PCR of *KIT* exon 17 followed by direct sequencing or sequencing of cloned RT-PCR products, the D816V *KIT* mutation was detected in the patient's bone marrow at the time of initial presentation in April 2003 and from peripheral blood after completion of 1 cycle of PKC412. However, these methods could not detect the D816V *KIT* mutation in the bone marrow after 2 cycles of therapy or from peripheral blood obtained at the completion of 3 cycles of PKC412 (during disease progression). All samples were re-evaluated with a more sensitive, semiquantitative mutation detection approach using DHPLC/Surveyor mismatch cleavage analysis (via WAVE DHPLC) and confirmatory sequencing. This method detected the D816V *KIT* mutation at a similar frequency in pretreatment and follow-up marrows, but an 80% decrease in the frequency of the mutation was observed in the peripheral blood through cycles 1 and 3 (Figure 2D), consistent with the marked decline in circulating mast cells (Figure 2B). Screening of the entire *KIT* coding region and splice junctions detected no additional mutations during treatment and at relapse.

#### Ex vivo analysis of KIT phosphorylation during PKC412 treatment

The densitometric signal obtained for phospho-KIT (eg, activated KIT) was, in each case, normalized to the densitometric signal

obtained for total KIT from the same sample. Since KIT is predominantly expressed on mast cells, this ratio controls for change in mast cell number from sample to sample. Because of insufficient bone marrow material for serial assessment of activated KIT, the patient's peripheral blood was used instead. The mean phospho-KIT/total KIT optical density declined during therapy (Figure 2C), by ~26% on day 7 of treatment, to ~53% on day 26 of treatment. There was no relative increase in KIT phosphorylation in the peripheral blood at the time of relapse (day 90).

PKC412 is an inhibitor of the family of FLT3, KIT, vascular endothelial growth factor receptor 2, platelet-derived growth factor receptor (PDGFR), and fibroblast growth factor receptor (FGFR) tyrosine kinases.<sup>22,24</sup> It is currently under evaluation in phase 2 clinical trials of AML associated with mutated FLT3.<sup>23</sup> PKC412 also demonstrated activity in a patient with stem cell myeloproliferative disorder (MPD) with the constitutively activated *ZNF198-FGFR1* fusion tyrosine kinase.<sup>24</sup> PKC412 was previously shown to be effective in a murine model of FIP1L1-PDGFR- $\alpha$ -induced myeloproliferative disease containing the imatinib-resistant T674I mutation.<sup>25</sup> Accordingly, we hypothesized that PKC412 might also be useful in SM disease related to the imatinib-resistant D816V *KIT* mutation.

We demonstrated that PKC412 could inhibit D816V KIT-transformed cells at an IC<sub>50</sub> of 30 nM to 40 nM, whereas these cells exhibit no significant inhibition by imatinib at concentrations of more than 1  $\mu$ M. These in vitro results prompted us to initiate a trial of PKC412 in our patient with mast cell leukemia. Our patient experienced substantial improvement in her performance status and mast cell-related symptoms. Her portal vein thrombosis recanalized, ascites decreased, and liver function improved. These findings constitute a partial response by proposed SM response criteria.<sup>27</sup> In addition, there was improvement in mast cell disease-related laboratory findings, including a marked decrease in peripheral blood mast cells and serum histamine level, associated with decreased KIT phosphorylation and D816V *KIT* mutation frequency. Persistent elevation of the serum tryptase level with PKC412 therapy may reflect ongoing infiltration of the bone marrow and other sites with mast cells, or the serum tryptase may have remained increased due to the patient's associated MDS/MPD.<sup>1,28</sup>

In contrast to the peripheral blood, there was minimal reduction of the burden of mast cells within the bone marrow. In phase 2 trials of FLT3 tyrosine kinase inhibitors (including PKC412) in AML, significant reductions in the percentage of peripheral blood blasts have frequently been observed without a corresponding decrease in bone marrow blasts.<sup>25</sup> These findings suggest that bone marrow mast cells and leukemic blasts may somehow be protected from this class of drugs by factors related to the bone marrow microenvironment.<sup>29</sup> If this is the case, then the identification of such factors and approaches for counteracting their effects would be important for developing new approaches for the effective and long-term treatment of these disorders.

PK results from this one patient suggest that PKC412 and its presumed active metabolite reached an effective level in the first week, when clinical responses were first observed, and the concentrations were maintained thereafter at generally the same levels between the end of months 1 through 3 of treatment (~500 ng/mL for PKC412 alone and ~1500 ng/mL for the sum of PKC412 and CGP62221). Although it cannot be concluded that the concentrations of PKC412 and CGP62221 were maintained above an effective level during this period, the quantity of D816V KIT-mutated DNA remained stable in the blood during cycles 2

and 3. Also, an increase of dosage to 75 mg three times a day on day 90 resulted in a rise in PK levels to troughs in the range of the first cycle of therapy, yet did not result in further improvement of the clinical findings, suggesting that inadequate PK was unlikely to be the cause for disease progression. The decreasing plasma concentrations are probably related to CYP3A4 enzyme induction by PKC412 and its metabolites.<sup>25</sup>

In this case, disease progression was related to transformation of the patient's MDS/MPD to AML. This is supported by the increasing percentage of myeloblasts and immature myeloid cells (but not mast cells) in the peripheral blood and bone marrow at the time of relapse. It is unknown whether this disease progression represents some form KIT-dependent or KIT-independent resistance of the patient's MCL to PKC412 therapy. However, screening of all exons of the *KIT* gene at the time of relapse did not reveal any other mutations that could have attributed to resistance to targeted therapy with PKC412. Also, there was no rebound increase in KIT phosphorylation at the time of relapse. We hypothesize that additional genetic mutations could have contributed to a clonal expansion of more phenotypically immature cells/blasts.

Taken together, these results indicate that PKC412 has promise in the treatment of aggressive forms of SM that are associated with the D816V *KIT* mutation. Akin to the more favorable experience with imatinib in chronic versus more advanced BCR-ABL-positive CML, it may also be useful to explore the efficacy of this agent in symptomatic patients with SM with less advanced disease. Use of PKC412 in this case provides the first proof-of-principle in humans that resistance of the D816V *KIT* tyrosine kinase to inhibition by imatinib can be circumvented with a structurally different small molecule inhibitor that targets the same protein.

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## References

- Valent P, Horny H-P, Li CY, et al. Mastocytosis (mast cell disease). In: Jaffe ES, Harris NL, Stein H, Vardiman JW, eds. World Health Organization (WHO) classification of tumours: pathology & genetics: tumours of haematopoietic and lymphoid tissues. Vol. 2. Geneva, Switzerland: WHO; 2001: 291-302.
- Valent P, Akin C, Sperr WR, et al. Diagnosis and treatment of systemic mastocytosis: state of the art. *Br J Haematol*. 2003;122:695-717.
- Galli SJ, Zeeb KM, Gelsinger EN. The KIT ligand, stem cell factor. *Adv Immunol* 1994;55:1-95.
- Nagata H, Worobec AS, Oh CK, et al. Identification of a point mutation in the catalytic domain of the proto-oncogene c-kit in peripheral blood mononuclear cells of patients who have mastocytosis with an associated hematologic disorder. *Proc Natl Acad Sci U S A*. 1995;92:10560-10564.
- Longley BJ, Tyrrell L, Lu SZ, et al. Somatic c-kit activating mutation in uveitis pigmentosa and aggressive mastocytosis: establishment of clonality in a human mast cell neoplasm. *Nat Genet*. 1996;12:312-314.
- Furuta T, Tajima T, Tono T, et al. Identification of mutations in the coding sequence of the proto-oncogene c-kit in a human mast cell leukemia cell line causing ligand-independent activation of c-kit product. *J Clin Invest*. 1993;92:1736-1744.
- Cassatane P, Calatayud-Vigneron N, Martin A, et al. Treatment of adult systemic mastocytosis with interferon- $\alpha$ : results of a multicentre phase II trial on 20 patients. *Br J Haematol*. 2002;119: 1090-1097.
- Hauswirth AW, Simonitsch-Klupp I, Ulfmann M, et al. Response to therapy with interferon- $\alpha$  and prednisolone in aggressive systemic mastocytosis: report of five cases and review of literature. *Leuk Res*. 2003;28:249-257.
- Kluin-Nelemans HC, Oldhoff JM, Van Doornaal JJ, et al. Cladribine therapy for systemic mastocytosis. *Blood*. 2003;102:4270-4276.
- Akin C, Brockow K, D'Ambrosio C, et al. Effects of tyrosine kinase inhibitor STI571 on human mast cells bearing wild-type or mutated c-kit. *Exp Hematol*. 2003;31:686-692.
- Ma Y, Zeng S, Metcalfe DD, et al. The c-KIT mutation causing human mastocytosis is resistant to STI571 and other KIT kinase inhibitors: kinases with enzymatic site mutations show different inhibitory sensitivity profiles than wild-type kinases and those with regulatory-type mutations. *Blood*. 2002;99:1741-1744.
- Pardani A, Elliott M, Reeder T, et al. Imatinib for systemic mast-cell disease. *Lancet*. 2003;362: 555-556.
- Propper DJ, McDonald AC, Man A, et al. Phase I and pharmacokinetic study of PKC412, an inhibitor of protein kinase C. *J Clin Oncol*. 2001;19: 1485-1492.
- Andersson J, Sjogren H, Mels-Kinshorn JM, Stenman G, Aman P, Kindblom LG. The complexity of KIT gene mutations and chromosome rearrangements and their clinical correlation in gastrointestinal stromal (pacemaker cell) tumors. *Am J Pathol*. 2002;160:15-22.
- Lilleberg SL, Durocher J, Sanders C, Walters K, Culver K. High sensitivity scanning of colorectal tumors and matched plasma DNA for mutations in APC, TP53, K-RAS, and BRAF genes with a novel dHPLC fluorescence detection platform. *Ann N Y Acad Sci*. 2004;1022:250-256.
- Gu P, Shandilya H, D'Alessio JM, O'Connor K, Durocher J, Gerard GF. Mutation detection using Surveyor nuclease. *Biochem*. 2004;8:702-707.
- Fumo G, Akin C, Metcalfe D, Neckers L. 17-Azaylmisino-17-demethoxygalegaramycin (17-AAG) is effective in down-regulating mutated, constitutively activated KIT protein in human mast cells. *Blood*. 2004;103:1078-1084.
- Cancer Therapy Evaluation Program: Common terminology criteria for adverse events, version 3.0. Bethesda, MD: National Cancer Institute, December 2003.
- Gronowey JD, Clark J, Adelsperger J, et al. Activation mutations of human c-kit resistant to imatinib are sensitive to the tyrosine kinase inhibitor PKC412. *Blood*. 2005;106:721-724.
- Casteran N, De Sepulveda P, Beaulieu N, et al. Signal transduction by several KIT extramembrane domain mutations. *Oncogene*. 2003;22:4710-4722.
- Ning ZQ, Li J, Arceri RJ. Signal transducer and activator of transcription 3 activation is required for Asp(816) mutant c-kit-mediated cytokine-independent survival and proliferation in human leukemia cells. *Blood*. 2001;97:3559-3567.
- Weisberg E, Boulton C, Kelly LM, et al. Inhibition of mutant FLT3 receptors in leukemia cells by the small molecule tyrosine kinase inhibitor PKC412. *Cancer Cell*. 2002;1:433-443.
- Fabbro D, Ruetz S, Bode S, et al. PKC412—a protein kinase inhibitor with a broad therapeutic potential. *Anticancer Drug Res*. 2000;15:17-28.
- Chen J, DeAngelis DJ, Kutok JL, et al. PKC412 inhibits the zinc finger 126-fibroblast growth factor receptor 1 fusion tyrosine kinase and is active in the treatment of stem cell myeloproliferative disorder. *Proc Natl Acad Sci U S A*. 2004;101:14479-14484.
- Stone RM, DeAngelis DJ, Klinev V, et al. Patients with acute myeloid leukemia and an activating mutation in FLT3 respond to a small-molecule FLT3 tyrosine kinase inhibitor, PKC412. *Blood*. 2005;105:54-60.
- Cooks J, Stover EH, Boulton CL, et al. PKC412 overcomes resistance to imatinib in a murine model of P1151-PDGFR $\alpha$ -induced myeloproliferative disease. *Cancer Cell*. 2003;3:459-469.
- Valent P, Akin C, Sperr WR, et al. Aggressive systemic mastocytosis and related mast cell disorders: current treatment options and proposed response criteria. *Leuk Res*. 2003;27:635-641.
- Sperr WR, Stehnbeger B, Wimsaats F, et al. Serum tryptase measurements in patients with myelodysplastic syndromes. *Leuk Lymphoma*. 2002;43: 1097-1105.
- Gilliland DG. FLT3 inhibitors in the treatment of AML. *Clin Adv Hematol Oncol*. 2004;2:67-110.

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## **PKC412 inhibits in vitro growth of neoplastic human mast cells expressing the D816V-mutated variant of KIT: comparison with AMN107, imatinib, and cladribine (2CdA) and evaluation of cooperative drug effects**

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# PKC412 inhibits in vitro growth of neoplastic human mast cells expressing the D816V-mutated variant of KIT: comparison with AMN107, imatinib, and cladribine (2CdA) and evaluation of cooperative drug effects

Karoline V. Gleixner, Matthias Mayerhofer, Karl J. Aichberger, Sophia Dardak, Karoline Sonneck, Alexandra Böhm, Alexander Gruze, Puchit Samorapomichit, Paul W. Manley, Dorian Fabbro, Winfried F. Pickl, Christian Sillaber, and Peter Valent

In most patients with systemic mastocytosis (SM), including aggressive SM and mast cell leukemia (MCL), neoplastic cells express the oncogenic KIT mutation D816V. KIT D816V is associated with constitutive tyrosine kinase (TK) activity and thus represents an attractive drug target. However, imatinib and most other TK inhibitors fail to block the TK activity of KIT D816V. We show that the novel TK-targeting drugs PKC412 and AMN107 counteract TK activity of D816V KIT and inhibit the growth of Ba/F3 cells with doxycycline-inducible expression of KIT

D816V as well as the growth of primary neoplastic mast cells and HMC-1 cells harboring this KIT mutation. PKC412 was a superior agent with median inhibitory concentration ( $IC_{50}$ ) values of 50 to 250 nM without differences seen between HMC-1 cells exhibiting or lacking KIT D816V. By contrast, AMN107 exhibited more potent effects in KIT D816V<sup>+</sup> HMC-1 cells. Corresponding results were obtained with Ba/F3 cells exhibiting wild-type or D816V-mutated KIT. The growth-inhibitory effects of PKC412 and AMN107 on HMC-1 cells were associated with induction of

apoptosis and down-regulation of CD2 and CD63. PKC412 was found to cooperate with AMN107, imatinib, and cladribine (2CdA) in producing growth inhibition in HMC-1, but synergistic drug interactions were observed only in cells lacking KIT D816V. Together, PKC412 and AMN107 represent promising novel agents for targeted therapy of SM. (Blood. 2006;107:752-759)

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## Introduction

Several emerging modalities for the treatment of myeloid neoplasms are based on novel drugs targeting critical tyrosine kinases (TKs) or downstream signaling molecules.<sup>1-5</sup> Systemic mastocytosis (SM) is a hematopoietic neoplasm that behaves as an indolent myeloproliferative disease in most patients, but it can also present as an aggressive disease (aggressive SM [ASM]) or even as a leukemia (mast cell leukemia [MCL]).<sup>6-11</sup> In patients with ASM and MCL, the response to conventional therapy is poor, and the prognosis is grave.<sup>6-12</sup> Therefore, a number of attempts have been made to identify novel targets for therapy in neoplastic mast cells (MCs) and to define new treatment strategies for these patients.<sup>9-12</sup>

In the majority of all patients with SM, including those who have ASM or MCL, the somatic KIT point mutation D816V (Asp816Val) is detectable in neoplastic (mast) cells.<sup>13-17</sup> This mutation is associated with ligand-independent phosphorylation

and activation of KIT and autonomous differentiation and growth of affected cells.<sup>17,18</sup> Based on this concept, the D816V-mutated variant of KIT is an attractive target of therapy.<sup>9,12,19</sup> Thus, a number of efforts have been made in recent years to identify suitable drugs that would inhibit the TK activity of KIT D816V.<sup>9,12,19-24</sup> The TK inhibitor imatinib (STI571), which is widely used in clinical hematology, has recently been found to counteract growth of neoplastic MCs exhibiting wild-type (wt) KIT or the rarely occurring F522C-mutated variant of KIT.<sup>20,23</sup> In addition, this drug was found to block the growth of neoplastic cells in patients who have SM associated with clonal eosinophilia and a *FIP1L1/PDGFR* fusion gene.<sup>24-26</sup> However, imatinib failed to inhibit the growth of neoplastic MCs harboring the KIT mutation D816V,<sup>20,22</sup> which points to a clear need for the identification and development of novel TK inhibitors that block KIT D816V and thus inhibit growth of neoplastic MCs in SM.

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K.V.G. performed research experiments on KIT expression and phosphorylation as well as cell growth and drug interactions, analyzed the data, and contributed by

drafting the article; M.M. and C.S. contributed by establishing vital new analytic tools (Ba/F3 cells with inducible expression of KIT), by analyzing data, and by drafting and critically reviewing the manuscript; K.J.A., S.D., A.G., and W.F.P. contributed by performing key laboratory experiments on cell growth and proliferation and by analyzing respective data; K.S. performed flow cytometry experiments; A.B. conducted experiments with primary bone marrow-derived cells; P.S. performed electron microscopy experiments as well as TUNEL assay experiments; P.W.M. and D.F. contributed essential new reagents (PKC412, AMN107, imatinib), and P.V. contributed by designing the study, establishing the research plan, providing logistic and budget support, and approving the data and the final version of the manuscript.

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In the current study, we show that the novel TK inhibitors PKC412<sup>27</sup> and AMN107<sup>27</sup> counteract the growth of neoplastic human MCs and Ba/F3 cells expressing KIT D816V. PKC412 appears to be the more potent compound in this regard. We also show that PKC412 and AMN107 cooperate in producing growth inhibition in neoplastic MCs. These data suggest that PKC412 and AMN107 may be novel promising targeted drugs for the treatment of mastocytosis.

## Patient, materials, and methods

### Reagents

The TK inhibitors imatinib, AMN107,<sup>27</sup> and PKC412<sup>27</sup> (all from Novartis Pharma, Basel, Switzerland) were used in this study. Stock solutions of AMN107 and PKC412 were prepared by dissolving in dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany). Recombinant human (rh) stem cell factor (SCF) was purchased from Strathmann Biotech (Hannover, Germany), RPMI 1640 medium and fetal calf serum (FCS) from PAA Laboratories (Pasching, Austria), L-glutamine and Iscove modified Dulbecco medium (IMDM) from Gibco Life Technologies (Gaithersburg, MD), <sup>3</sup>H-thymidine from Amersham (Buckinghamshire, United Kingdom), and propidium iodide from Sigma (St Louis, MO). Interferon- $\alpha$  (IFN- $\alpha$ ) was from Roche (Basel, Switzerland), 2-chlorodeoxyadenosine (cladribine [2CdA]) from Janssen Cilag (Tinsville, NJ), and interleukin-4 (IL-4) from PeproTech (Rocky Hill, NJ). The phycoerythrin (PE)-labeled monoclonal antibodies (mAbs) RPA-2.10 (CD2), WM15 (CD13), YB5.B8 (CD117), and N6B6.2 (CD164) were purchased from Becton Dickinson (San Jose, CA), and the PE-conjugated mAbs CLB-gran12 (CD63) and 97A6 (CD203c) from Immunotech (Marseille, France).

### HMC-1 cells expressing or lacking KIT-D816V

The human mast cell line HMC-1,<sup>28</sup> generated from a patient with MCL, was kindly provided by Dr J. H. Butterfield (Mayo Clinic, Rochester, MN). Two subclones of HMC-1 were used, namely, HMC-1.1 harboring the KIT mutation V560G but not the KIT mutation D816V<sup>20</sup> and a second subclone, HMC-1.2, harboring both KIT mutations, that is, V560G and D816V.<sup>20</sup> HMC-1 cells were grown in IMDM supplemented with 10% FCS, L-glutamine, and antibiotics at 37°C and 5% CO<sub>2</sub>. HMC-1 cells were rethawed from an original stock every 4 to 8 weeks and were passaged weekly. As control of "phenotypic stability," HMC-1 cells were periodically checked for (1) the presence of metachromatic granules, (2) expression of KIT, and (3) the down-modulating effect of IL-4 (100 U/mL, 48 hours) on KIT expression.<sup>29</sup>

### Ba/F3 cells with inducible expression of wt KIT or KIT D816V

The generation of Ba/F3 cells with doxycycline-inducible expression of wt KIT (Ton.Kit.wt) or KIT D816V has recently been described.<sup>30</sup> In brief, Ba/F3 cells expressing the reverse tet-transactivator<sup>31,32</sup> were cotransfected with pTRE2 vector (Clontech, Palo Alto, CA) containing KIT D816V cDNA (or wt KIT cDNA, both kindly supplied by Dr J. B. Longley, Columbia University, New York, NY) and pTK-Hyg (Clontech) by electroporation. Stably transfected cells were selected by growing in hygromycin and cloned by limiting dilution. In this study, the subclone Ton.Kit.D816V.27<sup>30</sup> was used in all experiments. As assessed by Western blotting, immunocytochemistry, polymerase chain reaction (PCR), and restriction fragment length polymorphism (RFLP) analysis,<sup>16</sup> expression of KIT-D816V can be induced in Ton.Kit.D816V.27 cells within 12 hours by exposure to doxycycline (1  $\mu$ g/mL).<sup>30</sup>

### Isolation of primary neoplastic MCs

Primary bone marrow (BM) MCs were obtained from a female patient (aged 54 years) with smoldering systemic mastocytosis (SSM), a distinct subvariant of SM characterized by involvement of multiple hematopoietic lineages and detection of KIT D816V in MC- and non-MC-lineage myeloid cells.<sup>33-36</sup> For control purpose, BM obtained from a patient suffering from

malignant lymphoma (without BM involvement) was analyzed. Both patients gave informed consent before BM puncture, in accordance with the Declaration of Helsinki. The BM aspirate was collected in syringes containing preservative-free heparin. Cells were layered over Ficoll to isolate mononuclear cells (MNCs). The MNC fractions were found to contain 5% MCs in the patient with SSM and fewer than 1% MCs in the control sample (normal BM). Cell viability was more than 90%. The presence of the KIT mutation D816V in BM MNCs in the patient with SSM was confirmed by reverse transcription-PCR (RT-PCR) and RFLP analysis.<sup>16</sup>

### Analysis of KIT phosphorylation by Western blotting

HMC-1 cells (10<sup>6</sup>/mL) and Ba/F3 cells (10<sup>6</sup>/mL) containing either wt KIT (Ton.Kit.wt) or KIT D816V (Ton.Kit.D816V.27) were incubated with PKC412 (1  $\mu$ M), AMN107 (1  $\mu$ M), imatinib (1  $\mu$ M), or control medium at 37°C for 4 hours. Prior to exposure to inhibitory drugs, Ton.Kit.wt cells and Ton.Kit.D816V.27 cells were incubated with doxycycline (1  $\mu$ g/mL) at 37°C for 24 hours to induce expression of KIT. In the case of Ton.Kit.wt cells, KIT phosphorylation was induced by adding rhSCF (100 ng/mL). Immunoprecipitation (IP) and Western blotting were performed as described.<sup>32</sup> In brief, cells were washed at 4°C and resuspended in RIPA buffer (1 mL buffer/10<sup>6</sup> cells) containing 50 mM Tris, 150 mM NaCl, 1% Nonidet P40 (NP-40), 0.25% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na<sub>2</sub>VO<sub>4</sub>. After incubation in RIPA buffer supplemented with proteinase inhibitor cocktail (Roche) for 30 minutes at 4°C, lysates were centrifuged. For IP, lysates from 10<sup>7</sup> cells were incubated with anti-KIT antibody SR1 (kindly provided by Dr V. Broady, University of Washington, Seattle)<sup>37</sup> or with anti-KIT antibody 1C1 (kindly provided by Dr H.-J. Bühring, University of Tübingen, Tübingen, Germany)<sup>38</sup> and protein G-Sepharose beads (Amersham) in IP buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 100 mM NaF, and 1% NP-40) at 4°C overnight. Beads were then washed 3 times in IP buffer. Lysates and immunoprecipitates were separated under reducing conditions by 7.5% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Protran, Schleicher & Schuell, Keene, NH) by blotting containing 25 mM Tris, 192 mM glycine, and 20% methanol at 4°C. Membranes were blocked for 1 hour in 5% blocking reagent (Roche) and were then incubated with anti-KIT antibody 1C1 or with antiphospho protein Ab4G10 (Upstate Biotechnology, Lake Placid, NY) at 4°C overnight. Antibody reactivity was made visible by sheep anti-mouse IgG antibody and Lumigen PS-3 detection reagent (both from Amersham), with CL-Xposure film (Pierce Biotechnology, Rockford, IL).

### Measurement of <sup>3</sup>H-thymidine uptake

To determine growth-inhibitory drug effects, HMC-1 cells and Ba/F3 cells containing either SCF-activated wt KIT (Ton.Kit.wt) or KIT D816V (Ton.Kit.D816V.27) were incubated with various concentrations of PKC412 (100 pM to 10  $\mu$ M), AMN107 (1 nM to 10  $\mu$ M), or imatinib (3 nM to 300  $\mu$ M) in 96-well culture plates (TPP, Trasadingen, Switzerland) at 37°C for 48 hours. In time-course experiments, HMC-1 cells were exposed to PKC412 (300 nM) for 1, 12, 24, 36, or 48 hours. In select experiments, HMC-1 cells (both subclones) were incubated with various concentrations of IFN- $\alpha$  (0.1-500 000 U/mL) or 2CdA (0.005-10  $\mu$ g/mL). Primary cells (BM cells from a patient with SSM and control BM) were cultured in control medium, PKC412 (50-500 nM), AMN107 (100 nM to 30  $\mu$ M), or imatinib (1  $\mu$ M) for 48 hours. After incubation, 1  $\mu$ Ci (0.037 MBq) <sup>3</sup>H-thymidine was added (37°C, 12 hours). Cells were then harvested on filter membranes (Packard Bioscience, Meriden, CT) in a Filtermate 196 harvester (Packard Bioscience). Filters were air-dried, and the bound radioactivity was counted in a  $\beta$  counter (Top-Count NXT, Packard Bioscience).

To determine potential additive or synergistic drug effects on growth of neoplastic MCs, HMC-1 cells (both subclones) were exposed to various combinations of drugs (PKC412, AMN107, imatinib, IFN- $\alpha$ , 2CdA) at a fixed ratio of drug concentrations. Drug interactions (additive, synergistic) were determined by calculating combination index values using an commercially available software (CalcuSyn; Biosoft, Ferguson, MO).<sup>39</sup> All experiments were performed in triplicate.

### Evaluation of apoptosis by conventional morphology and electron microscopy

The apoptosis-inducing effects of TK inhibitors were analyzed by morphologic examination, flow cytometry, and electron microscopy. In typical experiments, HMC-1 cells were incubated with various concentrations of PKC412 (500 nM to 1  $\mu$ M), AMN107 (50 nM to 10  $\mu$ M), imatinib (50 nM to 10  $\mu$ M) or control medium in 6-well culture plates (TPP) in IMDM containing 10% FCS at 37°C for 24 hours. The percentage of apoptotic cells was quantified on cytosin preparations stained with Wright-Giemsa stain. Apoptosis was defined according to conventional cytologic criteria (cell shrinkage, condensation of chromatin structure).<sup>40</sup>

To confirm apoptosis in HMC-1 cells, electron microscopy was performed as described.<sup>41,42</sup> Using HMC-1 cells (both subclones) exposed to PKC412 (500 nM, 900 nM, or 1  $\mu$ M), AMN107 (1  $\mu$ M), imatinib (1  $\mu$ M), or control medium for 24 hours. After incubation, cells were washed and fixed in 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.025%  $\text{CaCl}_2$  buffered in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 hour. Cells were then washed, suspended in 2% agar, and centrifuged. Pellets were postfixed with 1.3%  $\text{OsO}_4$  (buffered in 0.66 M collidine) and stained en bloc in 2% uranyl acetate and sodium maleate buffer (pH 4.4) for 2 hours. Pellets were then rinsed, dehydrated in alcohol series, and embedded in EPON 812. Ultrathin sections were cut and placed on gold grids. Sections were contrasted in uranyl acetate and lead citrate and viewed in a JEOL 1200 EX II transmission electron microscope (JEOL, Tokyo, Japan).

### Evaluation of apoptosis by TUNEL assay and flow cytometry

To confirm that apoptosis occurred in HMC-1 cells following a 24-hour exposure to either PKC412 (1  $\mu$ M), AMN107 (1  $\mu$ M), or imatinib (1  $\mu$ M), an *in situ* terminal transferase-mediated dUTP-fluorescence nick end-labeling (TUNEL) assay was used as reported.<sup>43,44</sup> In brief, cells were fixed in 1% formaldehyde at pH 7.4 at 0°C for 15 minutes. Cells were then treated with 70% ethanol (ice-cold) for 1 hour, washed, and incubated in terminal-transferase reaction solution containing  $\text{CoCl}_2$ , DNA deoxynucleoside-triphosphate, and biotin-16'-2'-deoxy-uridine-5'-triphosphate (Boehringer Mannheim, Mannheim, Germany) at 37°C for 10 minutes. After incubation, cells were washed and incubated with streptavidin fluorescein (10  $\mu$ g/mL; Boehringer Mannheim) at 37°C for 20 minutes. Cells were then washed and analyzed with a Nikon Eclipse E 800 fluorescence microscope (Tokyo, Japan).

For flow cytometric determination of apoptosis and cell viability, combined annexin V-propidium iodide staining was performed. HMC-1 cells were exposed to PKC412 (0.5, 1, and 2.5  $\mu$ M), AMN107 (0.5, 1, and 2.5  $\mu$ M), imatinib (0.5, 1, and 2.5  $\mu$ M), or control medium at 37°C for 24 hours. Thereafter, cells were washed and incubated with annexin V-fluorescein isothiocyanate (FITC; Alexis Biochemicals, San Diego, CA) in binding buffer containing HEPES (10 mM, pH 7.4), NaCl (140 mM), and  $\text{CaCl}_2$  (2.5 mM). Thereafter, propidium iodide (1  $\mu$ g/mL) was added. Cells were then washed and analyzed by flow cytometry on a FACScan (Becton Dickinson).

### Evaluation of expression of activation-linked surface antigens on HMC-1 cells

Expression of cell-surface antigens on HMC-1.2 cells was determined by flow cytometry after culture in control medium or medium supplemented with TK inhibitors (PKC412, 1  $\mu$ M; AMN107, 1  $\mu$ M; imatinib, 1  $\mu$ M) for 24 hours. In select experiments, various concentrations of PKC412 (50, 100, 250, 500, and 1000 nM) were applied. After incubation with drugs, cells were washed and subjected to single-color flow cytometry using PE-conjugated antibodies against MC antigens known to be overexpressed on neoplastic MCs (compared with normal MCs) or are expressed at an early stage of mastoplasia (CD2, CD13, CD36, CD117, CD164, CD203c).<sup>45-47</sup> Flow cytometry was performed on a FACScan (Becton Dickinson) as described.<sup>29</sup>

Approval was obtained from the Institutional Review Board (Department of Internal Medicine I, Division of Hematology and Hemostaseology, Medical University of Vienna) for all series of experiments of this study.

### Statistical analysis

To determine the significance in differences between proliferation rates, apoptosis, and surface expression levels after exposure of HMC-1 cells to inhibitors, the Student *t* test for dependent samples was applied. Results were considered statistically significant at *P* less than .05.

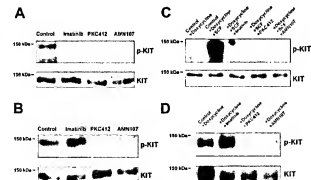
## Results

### Effects of PKC412 and AMN107 on TK activity of D816V-mutated KIT

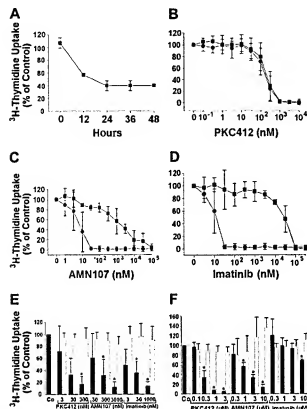
As assessed by IP and Western blotting, PKC412 (1  $\mu$ M) decreased phosphorylation of KIT in HMC-1.1 cells (expressing KIT V560G but not KIT D816V) as well as in HMC-1.2 cells harboring both mutations (Figure 1A-B). AMN107 (1  $\mu$ M) strongly reduced KIT phosphorylation in HMC-1.1 cells but showed only weak effects on KIT phosphorylation in HMC-1.2 cells at 1  $\mu$ M. Similarly, imatinib (1  $\mu$ M) reduced KIT phosphorylation in HMC-1.1 cells but did not inhibit KIT phosphorylation in HMC-1.2 cells (Figure 1A-B). We next examined effects of the TK inhibitors on Ba/F3 cells expressing either wt KIT (Ton.Kit.wt) or KIT D816V (Ton.Kit.D816V/27) after exposure to doxycycline. In Ton.Kit.wt cells, KIT appeared to be phosphorylated in the presence of SCF, whereas KIT was found to be constitutively phosphorylated in Ton.Kit.D816V/27 cells. As visible in Figure 1C, all 3 TK inhibitors (PKC412, AMN107, imatinib, each at 1  $\mu$ M) decreased the SCF-induced phosphorylation of KIT in Ton.Kit.wt cells. By contrast, only PKC412, and to a lesser degree AMN107, decreased KIT phosphorylation in Ton.Kit.D816V/27 cells. Imatinib (1  $\mu$ M) showed no effect on KIT phosphorylation in these cells (Figure 1D).

### Effects of TK inhibitors on <sup>3</sup>H-thymidine uptake in HMC-1 cells

In time-course experiments, maximum inhibitory effects of PKC412 on growth of HMC-1.1 and HMC-1.2 cells were seen after 36 to 48 hours. Figure 2A shows the time-dependent effect of PKC412 (300 nM) on growth of HMC-1.2 cells. As shown in Figure 2B-C, PKC412 and AMN107 counteracted <sup>3</sup>H-thymidine uptake in HMC-1.1 and HMC-1.2 cells in a dose-dependent manner. Interestingly,



**Figure 1.** Effects of TK inhibitors on KIT phosphorylation in neoplastic cells. (A-B) KIT phosphorylation in HMC-1.1 cells (A) and HMC-1.2 cells (expressing KIT D816V) (B) after incubation in control medium, imatinib (1  $\mu$ M), PKC412 (1  $\mu$ M), or AMN107 (1  $\mu$ M) for 4 hours. (C-D) KIT phosphorylation in Ton.Kit.wt cells (C) and Ton.Kit.D816V/27 cells (D) after incubation in control medium (control), imatinib (1  $\mu$ M), PKC412 (1  $\mu$ M), or AMN107 (1  $\mu$ M) for 4 hours. Prior to drug exposure, Ton.Kit.wt cells and Ton.Kit.D816V/27 cells were kept in doxycycline for 24 hours to induce expression of KIT. In case of the Ton.Kit.wt clone, cells were also exposed to SCF (100 ng/mL, 4 hours) to induce KIT phosphorylation (p-KIT). In all cells, immunoprecipitation was conducted using the anti-KIT mAb SR-1. Western blotting was performed using the antiphospho-mAb 4G10 for p-KIT detection and the anti-KIT mAb 1C1 for detection of total KIT protein.



**Figure 2.** Effects of PKC412 and AMN107 on proliferation of HMC-1 cells and Ba/F3 cells. (A) Time-dependent effects of PKC412 on  $^3\text{H}$ -thymidine uptake in HMC-1.2 cells. HMC-1.2 cells were incubated with control medium or PKC412 (300 nM) at 37°C and 5%  $\text{CO}_2$  for various time periods as indicated. After incubation,  $^3\text{H}$ -thymidine uptake was measured. Results are expressed as percent of control ( $^3\text{H}$ -thymidine uptake in control medium at each time point) and represent the mean  $\pm$  SD of triplicates. (B-D) Dose-dependent effects of TK inhibitors on  $^3\text{H}$ -thymidine uptake in HMC-1.1 cells (●) and HMC-1.2 cells (■) were incubated in control medium in the absence or presence of various concentrations of either PKC412 (B), AMN107 (C), or imatinib (D) at 37°C for 48 hours. After incubation,  $^3\text{H}$ -thymidine uptake was measured. Results are expressed as percent of control (100%) and represent the mean  $\pm$  SD from at least 3 independent experiments. (E-F) Effects of PKC412, AMN107, and imatinib on  $^3\text{H}$ -thymidine uptake in Ton.Kit cells. (E) Ton.Kit.wt cells were kept in IL-3-containing control medium (□) or were induced to express activated wt KIT by adding doxycycline (1  $\mu\text{g/mL}$ ) and SCF (in the absence of IL-3; ■). In both conditions, cells were exposed to either control medium (□) or were induced to express KIT D816V by adding doxycycline (1  $\mu\text{g/mL}$ ; ■), and then were exposed to either control medium (□) or various concentrations of PKC412, AMN107, or imatinib, as indicated, for 48 hours (37°C, 5%  $\text{CO}_2$ ). Thereafter,  $^3\text{H}$ -thymidine uptake was determined. Results are expressed as percent of control (□) and represent the mean  $\pm$  SD from 3 independent experiments. \* $P < .05$  compared with control.

the median inhibitory concentration ( $\text{IC}_{50}$ ) for the effects of PKC412 in these 2 subclones appeared to be in the same range (50–250 nM; Figure 2B). In contrast, the  $\text{IC}_{50}$  values for the effects of AMN107 on proliferation were significantly higher in HMC-1.2 cells (1–5  $\mu\text{M}$ ) compared to those found in HMC-1.1 cells (3–10 nM; Figure 2C). As expected, imatinib was effective only in HMC-1.1 cells ( $\text{IC}_{50}$ , 10–30 nM), whereas no significant effects of imatinib on HMC-1.2 cells were seen at pharmacologically relevant concentrations (Figure 2D), confirming previous data.<sup>20,22</sup> An interesting observation was that AMN107 was the most potent compound when comparing growth-inhibitory effects of the 3 drugs on HMC-1.1 cells exhibiting KIT V560G (Figure 2B-D).

### Effects of TK inhibitors on growth of Ba/F3 cells expressing wt KIT or KIT D816V

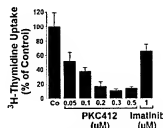
As shown in Figure 2E, all 3 TK inhibitors counteracted SCF-dependent growth of doxycycline-exposed (KIT-expressing) Ton.Kit.wt cells with  $\text{IC}_{50}$  values of 3 to 30 nM for PKC412, 30 to 300 nM for AMN107, and 3 to 30 nM for imatinib. By contrast, in Ton.Kit.D816V cells, only PKC412 ( $\text{IC}_{50}$ , 100–300 nM), and to a lesser degree AMN107 ( $\text{IC}_{50}$ , 1–3  $\mu\text{M}$ ), inhibited  $^3\text{H}$ -thymidine incorporation, whereas no significant effect was obtained with imatinib at pharmacologic concentrations (Figure 2F). None of the inhibitors counteracted growth of Ton.Kit.wt or Ton.Kit.D816V.27 cells in the absence of doxycycline, that is, in the absence of KIT (Figure 2E-F). In further control experiments, neither doxycycline (1  $\mu\text{g/mL}$ ) nor the TK inhibitors showed growth-inhibitory effects on non-transfected Ba/F3 cells (not shown).

### PKC412 and AMN107 counteract growth of primary neoplastic (mast) cells expressing KIT D816V

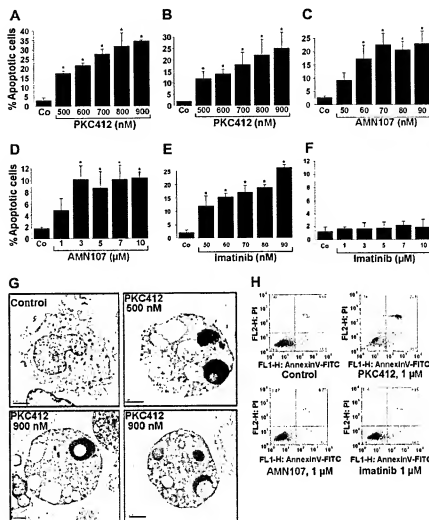
To confirm antiproliferative drug effects in SM, we examined primary neoplastic BM-derived MCs in a patient with SSM, a special subvariant of SM in which most myeloid cells (MCs as well as non-MC-lineage cells) exhibit KIT D816V. Although the purity of MCs was only 5%, most of the myeloid cells in this patient exhibited KIT D816V. In these neoplastic cells, PKC412 (and to a lesser degree AMN107, not shown) inhibited the spontaneous uptake of  $^3\text{H}$ -thymidine in a dose-dependent manner, whereas no significant effect was seen with imatinib at 1  $\mu\text{M}$  (Figure 3). In the control sample (normal BM), PKC412 showed no effect on  $^3\text{H}$ -thymidine uptake (not shown).

### PKC412 and AMN107 induce apoptosis in HMC-1 cells

To explore the mechanisms underlying the growth-inhibitory effects of PKC412 and AMN107, we analyzed morphologic and biochemical signs of apoptosis in HMC-1 cells after drug exposure. In these experiments, PKC412 induced apoptosis in both HMC-1 subclones (Figure 4A-B). AMN107 also induced apoptosis in both subclones, but the effect of this compound was much more pronounced in HMC-1.1 cells (Figure 4C) compared with HMC-1.2 cells (Figure 4D). Similarly, imatinib produced apoptosis in HMC-1.1 cells (Figure 4E), but showed no effect on HMC-1.2 cells (Figure 4F). The apoptosis-inducing effects of the drugs on HMC-1 cells were confirmed by electron microscopy. Again, all 3 drugs (each at 1  $\mu\text{M}$ ) induced apoptosis in HMC-1.1 cells, whereas in HMC-1.2, only PKC412 and, to a lesser degree AMN107, were



**Figure 3.** PKC412 down-regulates growth of primary neoplastic (mast) cells exhibiting D816V. Primary neoplastic BM cells exhibiting KIT D816V were isolated from a patient with SSM. Isolated cells were incubated in control medium (□) or with various concentrations of PKC412 or imatinib (1  $\mu\text{M}$ ) as indicated. Cell growth was quantified by measuring  $^3\text{H}$ -thymidine uptake. Results are expressed as percent of control (□, 100%) and represent the mean  $\pm$  SD of triplicates. In normal BM cells, no effects of PKC412 were seen (not shown).



**Figure 4.** Effects of TK inhibitors on apoptosis of HMC-1 cells. (A-F) HMC-1.1 cells (A,C,E) and HMC-1.2 cells (B,D,F) were cultured in the absence (Co) or presence of various concentrations of PKC412 (A-B), AMN107 (C-D), or imatinib (E-F), as indicated, for 24 hours. Thereafter, the percentages of apoptotic cells were quantified by light microscopy. Results represent the mean  $\pm$  SD of 3 independent experiments. \* $P < .05$  compared with control. (G) Electron microscopic examination of PKC412-induced apoptosis in HMC-1 cells. HMC-1.2 cells were incubated with control medium or PKC412 (500 nM or 900 nM as indicated) at 37°C for 24 hours. Then, cells were harvested and analyzed for ultrastructural signs of apoptosis. Whereas apoptotic cells were rarely seen in cultures kept with control medium, HMC-1.2 cells cultured in PKC412 frequently displayed signs of apoptosis including cell shrinkage, membrane ruffling, vacuolization, and condensation of the nuclear chromatin. Original magnification, 5000 $\times$ . Images were captured using a Gatan Bioscan Camera model 792 and Digital Micrograph acquisition software (Gatan, Pleasanton, CA). (H) HMC-1.2 cells were exposed to control medium (Control), PKC412 (1  $\mu$ M), AMN107 (1  $\mu$ M), or imatinib (1  $\mu$ M) at 37°C for 24 hours. Then, cells were examined for viability and apoptosis by combined propidium iodide (PI)/annexin V-FITC staining.

found to produce apoptosis. Figure 4G shows the apoptosis-inducing effect of PKC412 (24 hours) on HMC-1.2 cells. Finally, we were able to demonstrate the apoptosis-inducing effect of PKC412 in HMC-1 cells by combined annexin V/propidium iodide staining and flow cytometry (Figure 4H) as well as in a TUNEL assay (Figure 5). In both assays, PKC412 (1  $\mu$ M), and to a lesser degree AMN107 (1  $\mu$ M), induced apoptosis in HMC-1.2 cells, whereas imatinib showed no effects. In contrast, in HMC-1.1 cells, all 3 compounds induced apoptosis as assessed by the TUNEL assay (Figure 5).

#### PKC412 down-regulates expression of activation-linked cell-surface antigens on HMC-1 cells

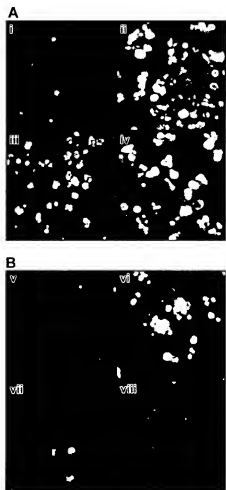
Several cell-surface antigens are typically (over)expressed on neoplastic MCs in SM.<sup>45,46</sup> Some of these molecules may be up-regulated directly by KIT D816V.<sup>30</sup> We therefore investigated whether PKC412, AMN107, or imatinib would influence expression of surface antigens on HMC-1.2 cells. Unstimulated cells expressed CD2, CD13, CD63, CD117, CD164, and CD203c, confirming previous data.<sup>45-47</sup> Incubation of HMC-1.2 cells with PKC412 resulted in a significant decrease in expression of CD2, CD63, and CD164 ( $P < .05$ ; Figure 6A). In contrast, no significant effects of PKC412 on expression of CD13 or CD203c were seen (Figure 6A). In the case of CD117/KIT, a slight decrease of

expression on HMC-1.2 cells was found on exposure to PKC412 (as well as on exposure to either AMN107 or imatinib), but the effects were not significant ( $P > .05$ ; Figure 6A). The effects of PKC412 on expression of CD2 and CD63 were dose dependent. Figure 6B shows the dose-dependent effect of PKC412 on expression of CD63 on HMC-1.2 cells. In contrast to PKC412, no significant effects of either AMN107 or imatinib on expression of CD antigens on HMC-1.2 cells were seen (Figure 6A).

#### PKC412 cooperates with other drugs in producing growth inhibition in HMC-1 cells

As assessed by <sup>3</sup>H-thymidine incorporation, PKC412 was found to cooperate with AMN107 in causing growth inhibition in HMC-1.1 and HMC-1.2 cells (Figures 7-8). In HMC-1.1 cells, the drug interaction was synergistic, whereas in HMC-1.2 cells, interactions were additive rather than synergistic (Figures 7-8). In addition, PKC412 and 2CdA, a drug used to treat ASM/MCL, inhibited growth of HMC-1.1 cells in a synergistic manner (Figure 8). By contrast, no synergistic effect of PKC412 and 2CdA on HMC-1.2 cells was seen. Similarly, AMN107 and imatinib produced synergistic inhibitory effects in HMC-1.1 cells (Figure 7), but not in HMC-1.2 cells carrying KIT D816V (Figure 8). No synergistic or additive effects on growth of HMC-1 cells were seen when





**Figure 5.** Apoptosis in HMC-1 cells assessed by TUNEL assay. HMC-1.1 cells (A) and HMC-1.2 cells (B) were incubated in control medium (i,v), PKC412, 1  $\mu$ M (ii,v), AMN107, 1  $\mu$ M (iii,vii), or imatinib, 1  $\mu$ M (iv,viii) at 37°C for 24 hours. Thereafter, cells were harvested and subjected to TUNEL assay. As visible, PKC412 produced apoptosis in most HMC-1.1 and HMC-1.2 cells, whereas AMN107 and imatinib showed potent apoptosis-inducing effects in HMC-1.1 cells (iii-iv), but not in HMC-1.2 cells exhibiting KIT D816V (vi-viii). Images were obtained using a Nikon Plan Apo 40  $\times$  1.0 numeric aperture oil objective. Images were acquired from FITC-labeled cells using a Hamamatsu high-resolution digital camera (model C4242-95; Hamamatsu, Japan) and HPD-CPX32 Microsoft Windows 95 software (Microsoft, Redmond, WA). Cytifluor (Agar Science, Stansted, United Kingdom) was used as imaging solution.

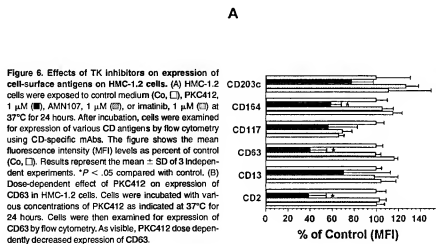
combining PKC412 and IFN- $\alpha$  or AMN107 and IFN- $\alpha$  (Figure 8). Single-drug effects ( $IC_{50}$ ) on growth of HMC-1.2 cells were: 2CdA, 10–20 ng/mL, and IFN- $\alpha$ , more than 100 U/mL.  $IC_{50}$  values for growth-inhibitory effects on HMC-1.1 cells were: 2CdA, 100–300 ng/mL, and IFN- $\alpha$ , more than 100 U/mL. A summary of cooperative drug effects is shown in Figure 8.

## Discussion

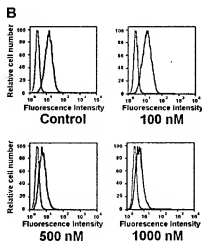
The somatic *KIT* mutation D816V is a gene defect that leads to constitutive activation of the *KIT* receptor, which is critically involved in growth of neoplastic MCs and thus in the pathogenesis of SM.<sup>13–17</sup> Therefore, recent attempts have focused on the identification and development of pharmacologic compounds that inhibit the TK activity of *KIT* D816V and thereby can inhibit growth of neoplastic MCs in patients with SM.<sup>8–12</sup> We here describe that the TK inhibitors PKC412, and to a lesser degree AMN107, inhibit TK activity of *KIT* D816V as well as growth of neoplastic human MCs carrying this particular *KIT* mutation. In addition, we show that both drugs cooperate with each other as well as with other targeted and conventional drugs in producing growth inhibition.

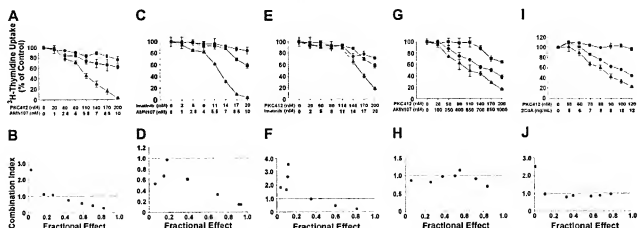
PKC412 is a staurosporine-related inhibitor of PKC and of several TKs including PDGFRA, FLT3, and *KIT*.<sup>5</sup> In this study, we show that PKC412 counteracts the growth of neoplastic human MCs and Ba/F3 cells expressing the D816V-mutated variant of *KIT*. With regard to Ba/F3 cells, our data are in line with the results of Growney et al.<sup>48</sup> Interestingly, the effective dose range for Ba/F3 cells was found to be the same as that found in HMC-1 cells. Another interesting observation was that the  $IC_{50}$  for the effects of PKC412 on the 2 subclones of HMC-1 (expressing or lacking *KIT* D816V) appeared to be in the same range. Finally, we were able to confirm growth-inhibitory effects of PKC412 for primary neoplastic (mast) cells expressing *KIT* D816V. Because the *KIT* mutation D816V is detectable in a majority of all patients with SM,<sup>13–17</sup> these data are of considerable importance. In fact, PKC412 seems to be the first TK inhibitor that counteracts growth of *KIT* D816V-bearing human MCs in the same way as MCs expressing wt *KIT*. It is also noteworthy in this regard that the inhibitory effects of PKC412 on *KIT* D816V<sup>+</sup> cells clearly exceeded the effects of AMN107. Thus, PKC412 seems to be a novel attractive targeted drug worthy to be considered for use in clinical trials in ASM or MCL.

Recent data suggest that AMN107 is a highly potent inhibitor of BCR/ABL TK activity.<sup>37</sup> It has also been reported that AMN107



**Figure 6.** Effects of TK inhibitors on expression of cell-surface antigens on HMC-1.2 cells. (A) HMC-1.2 cells were exposed to control medium (Co, □), PKC412, 1  $\mu$ M (■), AMN107, 1  $\mu$ M (▨), or imatinib, 1  $\mu$ M (▩) at 37°C for 24 hours. After incubation, cells were examined for expression of various CD antigens by flow cytometry using CD-specific mAbs. The figure shows the mean fluorescence intensity (MFI) levels as percent of control (Co, □). Results represent the mean  $\pm$  SD of 3 independent experiments. \* $P$  < .05 compared with control. (B) Dose-dependent effect of PKC412 on expression of CD63 in HMC-1.2 cells. Cells were incubated with various concentrations of PKC412 as indicated at 37°C for 24 hours. Cells were then examined for expression of CD63 by flow cytometry. As visible, PKC412 dose dependently decreased expression of CD63.





**Figure 7.** Synergistic drug effects on growth of HMC-1 cells. HMC-1 cells lacking KIT D816V (A-F) and HMC-1.2 cells exhibiting KIT D816V (G-J) were incubated with control medium or various combinations of drugs (at a fixed ratio) as indicated, at 37°C for 48 hours to determine cooperative antiproliferative effects. (A,C,E,G,I) After incubation with single drugs (A) PKC412 (■) and AMN107 (●); (C) imatinib (■) and AMN107 (●); (E) imatinib (■) and PKC412 (●); (G) PKC412 (■) and AMN107 (●); (I) PKC412 (■) and 2CdA (●) or drug combinations (A), cells were analyzed for <sup>3</sup>H-thymidine uptake. Results show <sup>3</sup>H-thymidine uptake as percentage of control (medium control, 100%) and represent the mean  $\pm$  SD of triplicates from one typical experiment (corresponding results were obtained in at least 2 other experiments for each drug combination). Images in the bottom row (B,D,F,H,J) show combination index values determined for each fraction affected according to the method described by Chou and Taliaferro<sup>39</sup> using CalcuSyn software. A combination index (CI) value of 1.0 indicates an additive effect, a CI greater than 1.0 indicates antagonism, and a CI of less than 1.0 indicates synergism.

inhibits TK activity of wt KIT.<sup>27</sup> We found that AMN107 exerts potent effects on HMC-1 cells carrying the *KIT* mutation V560G, but exhibits relatively weak effects on HMC-1 cells harboring both KIT V560G and KIT D816V. Similarly, AMN107 showed only weak effects on growth of Ba/F3 cells expressing the D816V-mutated variant of KIT. These data suggest that the *KIT* mutation D816V, but not the *KIT* mutation V560G, confers relative resistance against AMN107, although AMN107 still retains inhibitory effects on KIT D816V<sup>+</sup> cells compared with imatinib. The impressive antiproliferative effects of AMN107 on V560G<sup>+</sup> cells also suggest that this compound may be an attractive "drug candidate" for gastrointestinal stroma cell tumors, in which mutations at codon 560 of *KIT* have been reported.<sup>49</sup>

Several pharmacologic inhibitors targeting TK activity of pro-oncogenic molecules have recently been developed in clinical hematology.<sup>51,52,53</sup> The growth-inhibitory effects of these TK inhibitors on neoplastic cells (expressing the appropriate target) are usually associated with loss of TK activity and consecutive apoptosis. In the present study, we were also able to demonstrate that the growth-inhibitory effects of PKC412 on neoplastic MCs are associated with TK inhibition of (mutated) KIT as well as with apoptosis. The apoptosis-inducing effect of PKC412 was demonstrable by light and electron microscopy as well as by flow cytometry and in a TUNEL assay. As expected, both AMN107 and imatinib showed significant apoptosis-inducing effects on HMC-1.1 cells, but did not exhibit significant effects on HMC-1.2 cells.

	PKC412	AMN107	Imatinib	2CdA	IFN- $\alpha$
PKC412	■	+	+	+	—
AMN107	±	■	+	+	—
Imatinib	n.t.	±	■	n.t.	n.t.
2CdA	±	±	n.t.	■	n.t.
IFN- $\alpha$	—	—	n.t.	n.t.	■

**Figure 8.** Drug interactions on HMC-1.1 cells and HMC-1.2 cells. The effects of various drug combinations on growth of HMC-1.1 cells (upper right, □) and HMC-1.2 cells (lower left, ▢) were determined by <sup>3</sup>H-thymidine incorporation assay. Each drug combination was tested in at least 3 independent experiments. Drugs were applied at fixed ratio and resulting effects (the type of drug interaction) were determined by CalcuSyn software. Score: +, synergistic growth-inhibitory effect; ±, additive effect; —, less than additive (antagonistic) effect; n.t., not tested.

A number of cell-surface antigens are typically (over)-expressed on neoplastic MCs. Likewise, in contrast to normal MCs, neoplastic MCs in SM express CD2 and CD25.<sup>45,46</sup> We show that PKC412 down-regulates expression of CD2, CD63, and CD164 in HMC-1.2 cells exhibiting KIT D816V. A slight albeit insignificant effect of PKC412 on KIT expression was also seen. An interesting observation was that AMN107 failed to suppress expression of CD2 and CD63 on HMC-1.2 cells, which is probably due to its weaker effect on TK activity of KIT D816V compared with the effect of PKC412.

Recent data suggest that treatment of myeloid neoplasms with TK inhibitors as single agents may be insufficient to control the disease for prolonged time periods. This has been documented for the use of imatinib in advanced chronic myelogenous leukemia (CML)<sup>50,51</sup> and may also apply for patients with SM or MCL treated with imatinib or PKC412.<sup>52,53</sup> In these patients, drug resistance may occur. To overcome resistance, a number of pharmacologic strategies may be envisaged. One possibility is to apply drug combinations. We were therefore interested to learn whether PKC412 and AMN107 would exhibit additive or synergistic antiproliferative effects on HMC-1 cells. Indeed, our data show that PKC412 cooperates with imatinib and AMN107 in producing growth inhibition in both HMC-1.1 clones. Furthermore, PKC412 and 2CdA, a drug that has been described to counteract growth of neoplastic MCs in vivo (in aggressive) SM,<sup>54</sup> showed cooperative growth-inhibitory effects on HMC-1.1 and HMC-1.2 cells. However, drug interactions were synergistic on HMC-1.1 cells but not in HMC-1.2 cells. This may be explained by the relatively weak (AMN107) or absent (imatinib) effects of coupled drugs on KIT TK activity. Whether drug combinations consisting of PKC412 and other (targeted) drugs will be of clinical value in patients with ASM or MCL remains to be elucidated. Thus, so far, only a few agents with documented antiproliferative effects on neoplastic MCs in vivo in SM have been presented, and none of these drugs produce long-lasting complete remissions in patients with ASM or MCL.<sup>9,12,52-54</sup>

In summary, we show that PKC412 and AMN107 are novel promising drugs targeting KIT in SM. Whereas each of the 2 drugs exhibits a distinct pharmacologic profile with unique effects on mutated variants of KIT, a promising approach may be to combine both drugs with each other or with 2CdA to treat ASM or MCL.

## References

1. Kelly JT. Class III receptor tyrosine kinases: role in leukemogenesis. *Br J Haematol*. 2002;116:744-757.
2. Deininger MW, Druker BJ. Specific targeted therapy of chronic myelogenous leukemia with imatinib. *Pharmacol Rev*. 2003;55:401-423.
3. Pandanani A, Telford A. Imatinib targets other than bcr-abl and their clinical relevance in myeloid disorders. *Blood*. 2004;104:1931-1939.
4. Shah NP, Tran C, Lee FY, Chen P, Norris D, Sawyers CL. Overriding imatinib resistance with a novel ABL kinase inhibitor. *Science*. 2004;305:399-401.
5. Fabro D, Ruetz S, Bodis S, et al. PKC412—a protein kinase inhibitor with a broad therapeutic potential. *Anticancer Drug Des*. 2000;15:17-28.
6. Lennert K, Parvareesh MR. Mast cells and mast cell neoplasia: a review. *Histopathology*. 1979;3:349-365.
7. Metcalfe DD. Classification and diagnosis of mastocytosis: current status. *J Invest Dermatol*. 1991;96:23-45.
8. Valent P. Biology, classification and treatment of human mastocytosis. *Wien Klin Wochenschr*. 1996;108:385-397.
9. Valent P, Akin C, Sperr WR, et al. Diagnosis and treatment of systemic mastocytosis: state of the art. *Br J Haematol*. 2003;122:695-717.
10. Akin C, Metcalfe DD. Systemic mastocytosis. *Annu Rev Med*. 2004;55:419-432.
11. Telford A, Pandanani A. Clinical, genetic, and therapeutic insights into systemic mast cell disease. *Curr Opin Hematol*. 2004;11:58-64.
12. Valent P, Ghenadani M, Akin C, et al. On the way to targeted therapy of mast cell neoplasms: identification of molecular targets in neoplastic mast cells and evaluation of arising treatment concepts. *Eur J Clin Invest*. 2004;34:41-52.
13. Nagata H, Worobec AS, Oh CK, et al. Identification of a point mutation in the catalytic domain of the protooncogene c-kit in peripheral blood mononuclear cells of patients who have mastocytosis with an associated hematologic disorder. *Proc Natl Acad Sci U S A*. 1995;92:10560-10564.
14. Longley BJ, Tyrrell L, Lu SZ, et al. Somatic c-kit activating mutation in urticaria pigmentosa and aggressive mastocytosis: establishment of clonality in a human mast cell neoplasm. *Nat Genet*. 1996;12:312-314.
15. Longley BJ, Metcalfe DD, Tharp M, et al. Activating and dominant inactivating c-kit catalytic domain mutations in distinct forms of human mastocytosis. *Proc Natl Acad Sci U S A*. 1999;96:1609-1614.
16. Fritzsche-Polanz R, Jordan JH, Felix A, et al. Mutation analysis of C-KIT in patients with myelodysplastic syndromes without mastocytosis and cases of systemic mastocytosis. *Br J Haematol*. 2001;113:357-364.
17. Fager F, Ribadeau Dumas A, Leriche L, Valent P, Akin C. Kit and c-kit mutations in mastocytosis: a short overview with special reference to novel molecular and diagnostic concepts. *Int Arch Allergy Immunol*. 2002;127:110-114.
18. Furusue T, Tsujimura T, Tono T, et al. Identification of mutations in the coding sequence of the proto-oncogene c-kit in a human mast cell leukemia cell line causing ligand-independent activation of the c-kit product. *J Clin Invest*. 1993;92:1736-1744.
19. Telford A, Pandanani A. Systemic mastocytosis: current concepts and treatment advances. *Curr Hematol Rep*. 2004;3:197-202.
20. Akin C, Brockow K, D'Ambrone C, et al. Effects of tyrosine kinase inhibitor ST1571 on human mast cells bearing wild-type or mutated forms of c-kit. *Exp Hematol*. 2003;31:686-692.
21. Ma Y, Zeng S, Metcalfe DD, et al. The C-KIT mutation causing human mastocytosis is resistant to ST1571 and other KIT kinase inhibitors: kinases with enzymatic site mutations show different inhibitor sensitivity profiles than wild-type kinases and those with regulatory type mutations. *Blood*. 2002;99:1741-1744.
22. Frost MJ, Ferrao PT, Hughes TP, Ashman LK. Juxtamembrane mutant V560GKIT is more sensitive to imatinib (ST1571) compared with wild-type c-kit whereas the kinase domain mutant D816VKIT is resistant. *Mol Cancer Ther*. 2002;1:1115-1124.
23. Akin C, Fumo G, Yavuz AS, Lipsky PE, Neckers L, Metcalfe DD. A novel form of mastocytosis associated with a transmembrane c-kit mutation and response to imatinib. *Blood*. 2004;103:3222-3229.
24. Pandanani A, Ketterling RP, Brockman SR, et al. CHIC2 deletion, a surrogate for FIP1L1-PDGFR fusion, occurs in systemic mastocytosis associated with eosinophilia and predicts response to imatinib mesylate therapy. *Blood*. 2003;102:3093-3096.
25. Pandanani A, Elliott M, Reeder T, et al. Imatinib for systemic mast-cell disease. *Lancet*. 2003;362:555-558.
26. Pandanani A. Systemic mastocytosis: bone marrow pathology, classification, and current therapies. *Acta Haematol*. 2005;114:41-51.
27. Weisberg E, Manley PW, Breitenstein W, et al. Characterization of AMN107, a selective inhibitor of native and mutant Bcr-Abl. *Cancer Cell*. 2005;7:129-141.
28. Butterfield LH, Weiler D, Dewald G, Gleich GJ. Establishment of an immature mast cell line from a patient with mast cell leukemia. *Leuk Res*. 1988;12:345-355.
29. Silaber C, Strobl H, Bevec D, et al. IL-4 regulates c-kit proto-oncogene product expression in human mast and myeloid progenitor cells. *J Immunol*. 1991;147:4224-4228.
30. Mayerhofer M, Alchberger KJ, Florian S, et al. c-kit D816V provides a strong signal for myelomastocytic differentiation and cluster formation in murine Ba/F3 cells [abstract]. *Blood*. 2004;104:1418.
31. Deley QG, Baltimore D. Transformation of an interleukin-3-dependent hematopoietic cell line by the chronic myelogenous leukemia-specific P210bcr/abl protein. *Proc Natl Acad Sci U S A*. 1988;85:9312-9316.
32. Silaber C, Gebert P, Frank DA, Sattler M, Griffin JD. STA5 activation contributes to growth and viability in Bcr/Abi-transformed cells. *Blood*. 2002;95:2118-2125.
33. Valent P, Horny H-P, Escobedo L, et al. Diagnostic criteria and classification of mastocytosis: a consensus proposal. Conference Report of "Year 2000 Working Conference on Mastocytosis." *Leuk Res*. 2001;25:693-695.
34. Valent P, Horny H-P, Li CY, et al. Mastocytosis (mast cell disease). In: Jaffe ES, Harris NL, Stein H, Vardiman JW, eds. *World Health Organization (WHO) Classification of Tumours: Pathology & Genetics: Tumours of Haematopoietic and Lymphoid Tissues*. Lyon, France: IARC Press; 2001:291-302.
35. Yavuz AS, Lipsky PE, Yavuz S, Metcalfe DD, Akin C. Evidence for the involvement of a hematopoietic progenitor cell in systemic mastocytosis from single-cell analysis of mutations in the c-kit gene. *Blood*. 2002;100:661-665.
36. Valent P, Akin C, Sperr WR, Horny H-P, Metcalfe DD. Smouldering mastocytosis: a novel subtype of systemic mastocytosis with slow progression. *Int Arch Allergy Immunol*. 2002;127:137-139.
37. Broudy VC, Lin N, Zsebo KM, et al. Isolation and characterization of a monoclonal antibody that recognizes the human c-kit receptor. *Blood*. 1992;79:338-346.
38. Bühring HJ, Ashman LK, Gattell V, et al. Stem-cell factor receptor (c-kit) summary report (CD117). In: Schlossman SF, Boumell L, Gills W, et al, eds. *Leucocyte Typing V: White Cell Differentiation Antigens*. Vol 2. Oxford, United Kingdom: Oxford University Press; 1995:1882-1888.
39. Chou TC, Tsaiay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul*. 1982;22:75-135.
40. Van Cruchten S, Van Den Broeck W. Morphological and biochemical aspects of apoptosis, oncosis and necrosis. *Anat Hist Embryol*. 2002;31:214-223.
41. Schiede A, Samorapoomplit P, Füeder W, et al. Metal ion-induced toxic histamine release from human basophils and mast cells. *J Biomed Mater Res*. 1998;39:550-557.
42. Samorapoomplit P, Kiener HP, Schenbhaner GH, et al. Detection of tryptase in cytoplasmic granules of basophils in patients with chronic myeloid leukemia and other myeloid neoplasms. *Blood*. 2002;98:2580-2583.
43. Gorczyca W, Gong J, Darzynkiewicz Z. Detection of strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays. *Cancer Res*. 1993;53:1945-1951.
44. Walker PR, Carson C, Lebanc J, Sikorska M. Labeling DNA damage with terminal transferase: applicability, specificity, and limitations. *Methods Mol Biol*. 2002;203:3-19.
45. Escobedo L, Diaz-Agustín B, Belles C, et al. Utility of flow cytometric analysis of mast cells in the diagnosis and classification of adult mastocytosis. *Leuk Res*. 2001;25:563-570.
46. Valent P, Schenbhaner GH, Sperr WR, et al. Variable expression of activation-linked surface antigens on human mast cells in health and disease. *Immunol Rev*. 2001;173:74-81.
47. Ghanadani M, Hauswirth AW, Schenbhaner GH, et al. Detection of novel CD antigens on the surface of human mast cells and basophils. *Int Arch Allergy Immunol*. 2002;127:299-307.
48. Growney JD, Clark J, Adelsperger J, et al. Activation mutations in human c-kit resistant to imatinib are sensitive to the tyrosine kinase inhibitor PKC412. *Blood*. 2005;105:721-724.
49. Corfies CL, Fletcher JA, Heinrich MC. Biology of gastrointestinal stromal tumors. *J Clin Oncol*. 2004;22:3813-3825.
50. Cowan-Jacob SW, Guez V, Fendrich G, et al. Imatinib (ST1571) resistance in chronic myelogenous leukemia: molecular basis of the underlying mechanisms and potential strategies for treatment. *Mol Rev Med Chem*. 2004;4:285-299.
51. Weisberg E, Griffin JD. Resistance to imatinib (Gleevec): update on clinical mechanisms. *Drug Resist Update*. 2003;6:231-239.
52. Gottlieb J, Benze C, Growney JD, et al. Activity of the tyrosine kinase inhibitor PKC412 in a patient with mast cell leukemia with the D816V KIT mutation. *Blood*. 2005;106:2865-2870.
53. Droogendijk HJ, Kluin-Nelemans JC, van Daele PLA. Imatinib mesylate in the treatment of systemic mastocytosis: a phase I/II trial [abstract]. *Blood*. 2004;104:1516a.
54. Kluin-Nelemans HC, Oloff JM, Van Doornaal JJ, et al. Cladribine therapy for systemic mastocytosis. *Blood*. 2003;102:4270-4276.

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## Brief report

# Activation mutations of human c-KIT resistant to imatinib mesylate are sensitive to the tyrosine kinase inhibitor PKC412

Joseph D. Gowney, Jennifer J. Clark, Jennifer Adelsperger, Richard Stone, Doriano Fabbro, James D. Griffin, and D. Gary Gilliland

Constitutively activated forms of the transmembrane receptor tyrosine kinase c-KIT have been associated with systemic mast cell disease, acute myeloid leukemia, and gastrointestinal stromal tumors. Reports of the resistance of the kinase domain mutation D816V to the adenosine triphosphate (ATP)-competitive kinase inhibitor imatinib mesylate prompted us to characterize 14 c-KIT mutations reported in association with human hematologic malignancies for

transforming activity in the murine hematopoietic cell line Ba/F3 and for sensitivity to the tyrosine kinase inhibitor PKC412. Ten of 14 c-KIT mutations conferred Interleukin 3 (IL-3)-independent growth, c-KIT D816Y and D816V transformed cells were sensitive to PKC412 despite resistance to imatinib mesylate. In these cells, PKC412, but not imatinib mesylate, inhibited autophosphorylation of c-KIT and activation of downstream effectors signal transducer and tran-

scriptional activator 5 (Stat5) and Stat3. Variable sensitivities to PKC412 or imatinib mesylate were observed among other mutants. These findings suggest that PKC412 may be a useful therapeutic agent for c-KIT-positive malignancies harboring the imatinib mesylate-resistant D816V or D816Y activation mutations. (Blood. 2005;106:721-724)

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## Introduction

c-KIT is a member of the type 3 subclass of transmembrane receptor tyrosine kinases, characterized by 5 immunoglobulin-like domains in the extracellular region, a negative regulatory juxtamembrane (JM) domain and a split adenosine triphosphate-binding and phosphotransferase tyrosine kinase domain (reviewed in Broudy<sup>1</sup>). c-KIT activation by stem cell factor (SCF) promotes dimerization and transphosphorylation at tyrosine residues, resulting in downstream signaling events leading to cell growth. Mutations of c-KIT have been associated with hematopoietic and nonhematopoietic tumors, including systemic mast cell disease (SMCD),<sup>2-4</sup> acute myeloid leukemia (AML),<sup>5-8</sup> and gastrointestinal stromal tumors (GISTs).<sup>9-12</sup>

The tyrosine kinase inhibitor imatinib mesylate (Gleevec; Novartis Pharma AG, Basel, Switzerland) is efficacious in the majority of patients with GIST harboring c-KIT mutations.<sup>9,13</sup> c-KIT is most commonly activated in GIST tumors by small deletions in the JM that are thought to constitutively activate the tyrosine kinase by loss of autoinhibitory function.<sup>14</sup> All c-KIT JM deletion mutants tested thus far are sensitive to inhibition by

imatinib mesylate. These observations have suggested that imatinib mesylate might be of value in the treatment of SMCD or AML that also have activating mutations in c-KIT. However, the most common activating alleles in the context of SMCD and AML are the D816V and D816Y mutations in the c-KIT activation loop, and these mutations have been shown to be highly resistant to imatinib mesylate in vivo<sup>15</sup> and in vitro.<sup>16-19</sup>

PKC412 is a novel staurosporine-derived tyrosine kinase inhibitor that targets protein kinase C (PKC), kinase insert domain-containing receptor (KDR), vascular endothelial growth factor receptor-2 (VEGFR-2), platelet-derived growth factor receptor  $\alpha$  (PDGFR $\alpha$ ), Fms-like tyrosine kinase 3 (FLT3), and c-KIT.<sup>20</sup> PKC412 has previously been shown to be effective against the fusion protein Fip1-like 1 (FIP1L1)-PDGFR $\alpha$  with mutations in the kinase domain that are resistant to imatinib mesylate.<sup>21</sup> Since inhibitors of D816V/Y c-KIT mutations would potentially have therapeutic activity in SMCD and AML, we have evaluated the effectiveness of PKC412 against a panel of c-KIT mutations identified in patients with SMCD, GIST, and AML.

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One of the authors (J.D. Griffin) has declared a financial interest in Novartis Pharma AG, whose potential product, PKC412, was studied in the present work. One of the authors (D.F.) is employed by a company (Novartis Pharma AG) whose potential product was studied in the present work.

J. D. Gowney performed all of the molecular cloning, growth inhibition, and

signaling experiments; analyzed the data; and wrote the manuscript. J.J.C. sequenced the patient samples that identified the novel mutation. J.A. provided technical assistance with cell culture and Western blotting. R.S. was the treating physician for the patient with the novel mutation and participated in discussions of experimental design and interpretation of data. D.F. provided the pharmacologic reagents for this study and contributed to writing of the manuscript and experimental design. J. D. Griffin contributed to writing the manuscript and discussions of experimental design. D.G.G. participated in the design and analysis of the experiments and writing of the manuscript.

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## Study design

### Cell culture

A 2.9-kb *XhoI* cDNA fragment encoding human (hu)-c-KIT (lacking amino acids 510-513, GNNK) was cloned into the *XhoI* site of pCDNA3. c-KIT point mutations (Table 1) were generated (Transformer Site-Directed Mutagenesis Kit; Clontech, Palo Alto, CA) and subcloned into the *XhoI* site of MSCV-IRES-GFP and MSCV-PGK-NEO. The IL-3-dependent mouse pre-B-cell line Ba/F3 was maintained, and retrovirus was prepared, as previously described.<sup>22</sup> Ba/F3 cells were transduced with viral supernatant, expanded for 2 passages (with 10 ng/mL rmlL-3), then selected for IL-3-independent growth. Ba/F3 cells transduced with Neo-containing vectors were first selected in IL-3 with 100 mg/mL G418. IL-3-independent Ba/F3 cells transduced with wild-type (WT) c-KIT were selected with 20 ng/mL rhSCF.

Patient samples were collected following informed consent in accordance with the institutional review board (IRB)-approved Dana-Farber Cancer Institute protocol 01-206.

### Growth inhibition

Cells ( $1 \times 10^5$ ) in 100  $\mu$ L medium with or without IL-3 were incubated with various concentrations of imatinib mesylate or PKC412 in 96-well plates for 48 hours in triplicate.  $^3$ H-thymidine (1  $\mu$ Ci [0.037 MBq]) was added, and cells were incubated for 4 hours. Cells were harvested, and  $^3$ H-thymidine incorporation was determined. Growth inhibition was plotted as the ratio of the average  $^3$ H-thymidine incorporation in drug-treated wells relative to no-drug controls. IC<sub>50</sub>s for imatinib mesylate or PKC412 were calculated by sigmoidal curve fitting software (OriginLab, Northampton, MA).

### Immunoprecipitations

Cell lysates, immunoprecipitations, and immunoblotting were performed as previously described.<sup>22</sup> c-KIT was precipitated with 5  $\mu$ g anti-human c-KIT antibody (Assay Design Systems, Ann Arbor, MI), separated on a 7.5% denaturing polyacrylamide gel and transferred to nitrocellulose. Blots were probed with mouse anti-phosphotyrosine antibody 4G10 (Upstate, Charlottesville, VA), stripped, and reprobed with mouse anti-human c-KIT (E1): SC-17806 (Santa Cruz Biotechnology, Santa Cruz, CA). Whole-cell lysates (1/10th volume immunoprecipitate [IP]

input) were similarly separated and probed with rabbit anti-phospho-Stat5 (signal transducer and transcriptional activator 5) or rabbit anti-phospho-Stat3, stripped, and reprobed with rabbit anti-Stat5a or rabbit anti-Stat3 (Cell Signaling Technology, Beverly, MA).

To assess constitutive tyrosine kinase autophosphorylation, cells were grown in RPMI plus 10% fetal calf serum (FCS), and 4 mg cell lysate was immunoprecipitated. To assess PKC412 inhibition of c-KIT autophosphorylation, cells were washed 3 times in RPMI without FCS or IL-3, then incubated with or without IL-3 and PKC412 (0-500 nM) for 4 hours. WT Ba/F3 cells were starved of IL-3 and serum for 4 hours prior to addition of IL-3 and PKC412. Cell equivalents ( $1 \times 10^7$ ) of cell lysate were immunoprecipitated.

## Results and discussion

The IL-3-dependent murine pre-B-cell line Ba/F3 was transduced with retroviruses expressing WT c-KIT or 1 of 14 c-KIT mutants and selected for IL-3-independent growth (Table 1; Figure 1A). Ten of 14 mutant forms of c-KIT transformed Ba/F3 cells to IL-3-independent growth in the absence of rhSCF. Transforming mutations mapped to the extracellular domain (delTYD (417-419) + I,<sup>3</sup> delTYD (417-419) + RG,<sup>4</sup> and A502Y503dup (AYdup)<sup>10</sup>), the juxtamembrane domain (V559D,<sup>11</sup> V560G,<sup>2</sup> and delV559V560 (delVV)<sup>11</sup>), and the kinase domain (R634W, D816Y,<sup>3</sup> D816V,<sup>2</sup> and N822K<sup>7</sup>). This is the first report of the kinase domain mutation R634W, identified from a patient with chronic myelomonocytic leukemia. Consistent with a report that E839K inactivates the kinase domain,<sup>12</sup> E839K did not transform Ba/F3 cells to IL-3-independent growth. K642E has been shown to be constitutively phosphorylated in a GIST-derived cell line<sup>10,23</sup> but did not transform Ba/F3 cells in this study. D820G<sup>4</sup> and V530I,<sup>5</sup> each identified in a single patient, also did not transform Ba/F3 cells.

Immunoprecipitated c-KIT was constitutively phosphorylated in all transformed cell lines and Ba/F3 cells expressing WT c-KIT grown with 20 ng/mL rhSCF (Figure 1B). We observed differential phosphorylation of 2 c-KIT bands of approximately 160 and 145 kDa, representing the fully glycosylated cell surface receptor, and

Table 1. Imatinib mesylate and PKC412 IC<sub>50</sub>s of transforming c-KIT mutations

Cell line/c-KIT mutation	Reference	Location*	Transforming	Imatinib mesylate IC <sub>50</sub> , nM†	PKC412 IC <sub>50</sub> , nM‡
Ba/F3	—	—	—	7 274	304
kit WT and rhSCF, without IL-3	—	NA	—	237	138
kit WT and rhSCF, with IL-3	—	NA	—	24 360	345
delTYD(417-419) and 1	Garl et al, 1999 <sup>9</sup>	EC	Yes	226	217
delTYD(417-419) and RG	Garl et al, 1999 <sup>9</sup>	EC	Yes	45	95
A502Y503dup	Lux et al, 2000 <sup>10</sup>	EC	Yes	196	206
V530I	Garl et al, 1999 <sup>9</sup>	TM	No	ND§	ND§
V559D	Hirota et al, 1998 <sup>11</sup>	JM	Yes	11	265
V560G	Furutsu et al, 1993 <sup>2</sup>	JM	Yes	53	82
delV559V560	Hirota et al, 1998 <sup>11</sup>	JM	Yes	15	146
R634W	Current study	K	Yes	198	85
K642E	Lux et al, 2000 <sup>10</sup>	K	No	ND§	ND§
D816Y	Tsujimura et al, 1995 <sup>3</sup>	K	Yes	840	33
D816V	Furutsu et al, 1993 <sup>2</sup>	K	Yes	10 651	44
D820G	Pignoni et al, 1997 <sup>4</sup>	K	No	ND§	ND§
N822K	Beghini et al, 2002 <sup>7</sup>	K	Yes	139	59
E839K	Longley et al, 1999 <sup>12</sup>	K	No	ND§	ND§

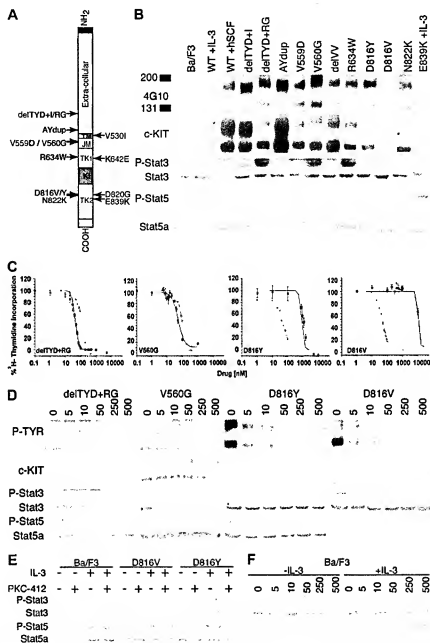
IC<sub>50</sub> indicates inhibitory concentration 50%; WT, wild type; rhSCF, recombinant human stem cell factor; IL-3, interleukin 3; NA, not applicable; EC, extracellular domain; TM, transmembrane domain; ND, not determined; JM, juxtamembrane; K, kinase domain.

\*Domain of c-KIT containing mutation.

†Data are representative of 2 or more experiments performed in triplicate.

‡IL-3 rescued PKC412 growth inhibition (data not shown).

§The indicated mutants did not transform Ba/F3 cells to factor independence. Thus, we were unable to test the ability of kinase inhibitors to inhibit growth in the absence of IL-3.



**Figure 1.** Imatinib mesylate resistant c-KIT mutations are sensitive to PKC412. (A) Schematic of the c-KIT protein indicating the relative location of structural and functional domains, as well as location of 14 c-KIT mutations evaluated. The JM delTV mutation occurs at V559/V560. ■ indicates amino terminal signal peptide. Other domains of c-KIT are indicated as follows: □, extracellular domain; TM, transmembrane domain; JM, juxtamembrane domain; TK1, nucleotide binding subdomain of split kinase domain; KI, kinase insert domain; and TK2, catalytic subdomain of split kinase domain. (B) Ba/F3-transforming c-KIT mutations are constitutively phosphorylated and phosphorylate Stat3 and Stat5. Blots from top to bottom: anti-phosphotyrosine Western blot of Ba/F3 lysates immunoprecipitated with anti-c-KIT; anti-c-KIT Western blot of immunoprecipitates in top blot; anti-phospho-Stat3 Western blot of whole-cell lysates (WCLs) from IP in top blot; anti-Stat3 Western blot of same WCLs; anti-phospho-Stat5 Western blot of WCLs from IP in top blot; and anti-Stat5a Western blot of the same WCLs. (C) Representative growth curves of delTYD+RG, V560G, D816Y, and D816V transduced Ba/F3 cell lines. Plotted is the percentage  $\pm$  SD of <sup>3</sup>H-thymidine incorporation of drug-treated cells relative to no-drug controls. Cells were treated with imatinib mesylate (black) or PKC412 (red). Cells were grown in the absence of IL-3 or rhSCF. (D) Dose response of PKC412 inhibition of c-KIT, Stat3, and Stat5 phosphorylation. Shown are data from delTYD+RG, V560G, D816Y, and D816V transduced Ba/F3 cells. Blots are as in panel B. Cells were grown in the absence of IL-3 and the indicated concentration of PKC412 (nM) for 4 hours. (E) PKC412 does not inhibit IL-3-induced Stat5 phosphorylation. Blots from top to bottom: anti-phospho-Stat3 Western blot of WCLs from indicated cell lines with or without IL-3 (10 ng/mL) and PKC412 (250 nM/mL), for which Ba/F3 cells were starved of IL-3 and serum for 4 hours prior to addition of IL-3 and PKC412; anti-Stat3 Western blot of WCLs in top blot; anti-phospho-Stat5 Western blot of WCLs as in top blot; and anti-Stat5a Western blot of same WCLs. (F) Dose response of Stat5 phosphorylation to PKC412 in Ba/F3 cells. Blots are as for panel E. Ba/F3 cells were starved of IL-3 and FCS for 4 hours, then incubated with or without IL-3 (10 ng/mL) in the presence of the indicated concentration of PKC412 for 4 hours.

incompletely processed internalized forms of c-KIT, respectively. Our findings confirm reports that Stat3 is aberrantly phosphorylated by D816V and other c-KIT-activating mutations.<sup>24,25</sup> Stat5 was constitutively phosphorylated by all c-KIT-activating mutations grown in the absence of IL-3.

The effect of imatinib mesylate or PKC412 on cell growth of c-KIT-expressing cell lines was evaluated in a <sup>3</sup>H-thymidine incorporation assay (Table 1; Figure 1C; data not shown). Consistent with previous reports, D816V was resistant to imatinib mesylate ( $IC_{50}$  > 10 000 nM).<sup>16-19</sup> D816Y was also observed to be

resistant to imatinib mesylate ( $IC_{50}$  > 800 nM). Four mutations, delTYD + 1, AYdup, R634W, and N822K, showed  $IC_{50}$ s for imatinib mesylate similar to Ba/F3-WT-c-KIT + 20 ng/mL rhSCF (100-300 nM). As previously reported, V560G<sup>19</sup> was hypersensitive to imatinib mesylate, as were the mutations delTYD + RG, V559D, and delVV ( $IC_{50}$ s < 100 nM).

PKC412 inhibited growth of all c-KIT-transformed Ba/F3 lines, with  $IC_{50}$ s segregating into 2 groups. The imatinib mesylate-resistant D816V and D816Y mutants, as well as delTYD + RG, V560G, R634W, and N822K, were more sensitive to PKC412 ( $IC_{50}$ s, 33-95 nM) than was the wild-type receptor stimulated with rhSCF ( $IC_{50}$ , 138 nM). The remaining mutations had higher  $IC_{50}$ s for PKC412 (146-265 nM).

The level of tyrosine autophosphorylation of the imatinib mesylate-resistant D816V and D816Y c-KIT mutants, as well as phosphorylation of Stat3 and Stat5, was dose dependent on PKC412 over a wide concentration range (5-500nM) and correlated with inhibition of cell growth (Table 1; Figure 1D). Similar findings were observed for representative imatinib mesylate-sensitive extracellular (delTYD + RG) and JM (V560G) mutants. Although Stat5 phosphorylation

by D816V and D816Y was inhibited by PKC412 (Figure 1D-E), an increased level of Stat5 phosphorylation induced by stimulation with IL-3 induction was not inhibited in these cells (Figure 1E). IL-3 did not rescue PKC412 inhibition of phosphorylation of Stat3 (Figure 1E) and did not inhibit IL-3-induced Stat5 phosphorylation in WT Ba/F3 Cells (Figure 1E-F).

These data indicate that among the reported c-KIT mutations in human hematologic malignancies, 10 of 14 confer factor independent growth to Ba/F3 cells. There is variable sensitivity of these mutations to inhibition by imatinib mesylate or PKC412. Notably, however, the most common mutations D816V and D816Y are highly resistant to imatinib mesylate but sensitive to inhibition by PKC412. These findings suggest that PKC412 may be an effective therapeutic agent for treatment of patients with certain imatinib mesylate-resistant c-KIT mutations.

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## References

- Broudy VC. Stem cell factor and hematopoiesis. *Blood*. 1997;90:1345-1364.
- Furuta T, Tsujimura T, Tono T, et al. Identification of mutations in the coding sequence of the proto-oncogene c-kit in a human mast cell leukemia cell line causing ligand-independent activation of c-kit product. *J Clin Invest*. 1993;92:1736-1744.
- Tsujimura T, Furuta T, Morimoto M, et al. Substitution of an aspartic acid results in constitutive activation of c-kit receptor tyrosine kinase in a rat mast cell line RBL-2H3. *Int Arch Allergy Immunol*. 1995;108:377-385.
- Pignoni JM, Giraudier S, Duquesnoy P, et al. A new c-kit mutation in a case of aggressive mast cell disease. *Br J Haematol*. 1997;96:374-376.
- Beghini A, Petronzoni P, Ripamonti CB, et al. c-KIT mutations in core binding factor leukemias. *Blood*. 2000;95:726-727.
- Beghini A, Larizza L, Calzoli R, Morra E. c-KIT activating mutations and mast cell proliferation in human leukemia. *Blood*. 1998;92:701-702.
- Beghini A, Magnani L, Ripamonti CB, Larizza L. Amplification of a novel c-KIT activating mutation Asn(822)-Lys in the Kasumi-1 cell line: a (t(8;21)) c-KIT mutant model for acute myeloid leukemia. *Hematol J*. 2002;3:157-163.
- Gari M, Goodvive A, Wilson G, et al. c-KIT proto-oncogene exon 8 in-frame deletion plus insertion mutations in acute myeloid leukaemia. *Br J Haematol*. 1999;105:804-809.
- Duensing A, Heinrich MC, Fletcher DC, Fletcher JA. Biology of gastrointestinal stromal tumors: KIT. *Cancer Invest*. 2004;22:106-116.
- Lux ML, Rubin BP, Blase TL, et al. KIT extracellular and kinase domain mutations in gastrointestinal stromal tumors. *Am J Pathol*. 2000;156:791-795.
- Hirota S, Isozaki K, Moriyama Y, et al. Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. *Science*. 1998;279:577-580.
- Longley BJ Jr, Metcalfe DD, Thrup M, et al. Activating and dominant inactivating c-KIT catalytic domain mutations in clinical forms of human mastocytosis. *Proc Natl Acad Sci U S A*. 1999;96:1809-1814.
- Demetri GD, von Mehren M, Blanke CD, et al. Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. *N Engl J Med*. 2002;347:472-480.
- Heinrich MC, Corless CL, Demetri GD, et al. Kinase mutations and imatinib response in patients with gastrointestinal stromal tumor. *J Clin Oncol*. 2003;21:4342-4348.
- Pardanani A, Elliott M, Reeder T, et al. Imatinib for systemic mast-cell disease. *Lancet*. 2003;362:535-536.
- Ma Y, Zeng S, Metcalfe DD, et al. The c-KIT mutation causing human mastocytosis is resistant to STI571 and other KIT kinase inhibitors: kinases with enzymatic site mutations show different inhibitor sensitivity profiles than wild-type kinase and those with regulatory-type mutations. *Blood*. 2002;99:1741-1744.
- Zemall U, De Sepulveda P, Feger F, et al. Effect of tyrosine kinase inhibitor STI571 on the kinase activity of wild-type and various mutated c-kit receptors found in mast cell neoplasms. *Oncogene*. 2003;22:660-664.
- Akin C, Brockow K, D'Ambrasio C, et al. Effects of tyrosine kinase inhibitor STI571 on human mast cells bearing wild-type or mutated c-kit. *Exp Hematol*. 2003;31:686-692.
- Frost MJ, Ferraro PT, Hughes TP, Ashman LK. Juxtamembrane mutant V560GKIT is more sensitive to imatinib (STI571) compared with wild-type c-kit whereas the kinase domain mutant D816VKIT is resistant. *Mol Cancer Ther*. 2002;1:1115-1124.
- Fabbro D, Ruetz S, Bods S, et al. PKC412—a protein kinase inhibitor with a broad therapeutic potential. *Anticancer Drug Des*. 2000;15:17-28.
- Cools J, Slusher EH, Boulton CL, et al. PKC412 overcomes resistance to imatinib in a murine model of FIP1L1-PDGFRalpha-induced myeloid proliferative disease. *Cancer Cell*. 2003;3:459-469.
- Stember DW, Tomasson MH, Carroll M, et al. The TEL/PDGFRbeta fusion in chronic myelomonocytic leukemia signals through STAT5-dependent and STAT5-independent pathways. *Blood*. 2001;98:3390-3397.
- Tuveson DA, Willis NA, Jacks T, et al. STI571 inactivation of the gastrointestinal stromal tumor c-KIT oncoprotein: biological and clinical implications. *Oncogene*. 2001;20:5054-5058.
- Castellan M, De Sepulveda P, Beslu N, et al. Signal transduction by several KIT juxtamembrane domain mutations. *Oncogene*. 2003;22:4710-4722.
- Ning ZQ, Li J, Arcadi RJ. Signal transducer and activator of transcription 3 activation is required for Asp(816) mutant c-KIT-mediated cytokine-independent survival and proliferation in human leukemia cells. *Blood*. 2001;97:3559-3567.



RELATED PROCEEDINGS APPENDIX

Appellant is not aware of any related appeals of interferences. So Appellant has no information regarding related proceedings to submit.